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- METHOD OF QUANTIFYING GLYCOSYLATED PROTEIN USING REDOX REACTION AND QUANTIFICATION ICIT
- (67) The present invention provides a method of measuring a glycated protein in a sample using a redex reaction, by which the glycated protein can be measured accurately with high sensitivity. In order to remove a glyceted amino ecid present in the comple other than the plycated protein, the plycated amino acid is degraded in advance by causing a fructosyl amine acid exidaze to and thereon, and thereafter, a fructoryl amino acid oxi-dase is caused to act on the glycated protein in the presence of a tetrazolium compound and sodium exide to cause a redox reaction. The amount of the glycosted protein is determined by measuring the radax reaction. As the glyceted protein, glyceted homoglobin is preferable.

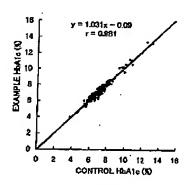


FIG. 1

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Description

Technical Floid

[0001] The present invention relates to a method of measuring a glycated protein using a radox reaction and to a

Background Art

- [B002] Among various glycated proteins, glycated homoglobin (glycated Hb) in blood serves as an important indicator in the disgnosis, treatment, etc. of disbetes, because it reflects the patients pest history of blood glucese levels.

 [0003] Measurement of such glycated Hb has been carried out, for exemple, by high parformance floud chromatography (MPLC), a minicolumn method, an immunoassay, a dye method, or the like. According to these methods, the ratio of glycated His to total His or the amount of glycated His can be determined. However, the above-described HPLC has a problem in that, for exemple, it requires a dedicated apparatus for measuring glycated His, and the immunos seal the described his and the immunos seal the described his seal that the described his seal to the described his seal to the described his seal that the descri and the dye method have problems in thei, for example, cells used for measurement might be contaminated and the mossurement constituty might not be sufficient.
 - [0004] On this account, measurement of a glyceted protein such as glyceted Hb using a redox reaction caused by an enzyme has been utilized in applications such as biochamical analyses and clinical tests, because it can be carried out easily without using a special monsuring apparetus.
- Goods Such measurement of glycarion have an a reaction is carried out, for example, in the following marrier.

 First, a sample containing glycated Hib is trusted with a fructosyl amino acid oxidese (herdinafter referred to as "FACO") so that the FACO acts on a glycarion site of the glycated Hb, thereby carriing pydrogen paraside to be formed. The amount of the hydrogen peroxide corresponds to the amount of the glycated Hb. Subsequently, to the sample treated with the FAOD, a peroxideae (heroinater reterned to as "POD") and a reducing egent are author so that a redox reaction occurs between the hydrogen paradde and the reducing agent with the POD as a catalyst. At this lime, when a reducing egent that develops color when it is exidized is used, the amount of the hydrogen perexide can be determined by measuring the color developed. As a result, the amount of the givested Hb in the sample can be determined.

30 Disclosure of Invention

- [000s] However, such conventional methods do not exhibit sufficient measurement sensitivity. Furthermore, with
- [0008] However, such conventional methods do not exhibit sufficient measurement sensitivity. Furthermore, with regard to measurement accuracy, there have been problems in that more hydrogen perceide may be formed than corresponds to glycabed his actually contained in a sample and that, depending on the patient, the measured value of glycated Hb may loop up temporarily. Therefore, further improvement in the measured securecy is desired.

 [0007] Therefore, it is an object of the present invention to provide a method of measuring a glycated protein in a sample using a redex reaction, by which the glycated protein can be measured accurately with high sometivity. Furthermore, it is another object of the present invention to provide a measuring kit to be used in the method, depable of architecture accuracy and measurement sensitivity and having a system operability. achieving excellent measurement accuracy and measurement sensitivity and having excellent operability.
- [DODG] In order to achieve the above object, the present invention provides a motified of measuring an amount of a glycated protein in a sample, including: enursing a FACO to act on a glycated amino acid present in the sample other then the grycated protein so as to remove the glycated contro acid by degrading it, then causing a FACO to act on the glycated protein to cause a redox reaction in the processes of a latracellum compound and sodium exide; and measuring
- gyested protein to determine the amount of the gyested protein.

 (1000s) It is to be noted that "FAOD" merely is a generic name and the substrate thereof is not limited to givested.
- [0009] It is to be noted that "FACID" merely is a generic name and the substrate thereof is not [imited to glycated fining acids. For example, FAODs act also on glycated proteins and glycated peptides, Herefinian, a FAOD used for degrading the glycated amine sold is referred to se a "degraded in FAOD" and a FAOD entered to set an the glycated protein to measure it is referred to set a "neasurament FAOD" in the present invention.

 [0010] The inventors of the present invention have conducted in-depth research to improve the accuracy of the measurement and finally found out the following fact. In whice blood, not only a glycated protein but also a free glycated protein but also a few glycated amine acid is present inherently. FAODs also set on such a glycated amine sold. Thus, when a glycated protein is measured using a FAOD as described above, a redox reaction occurs not only between the glycated protein and the FAOD is deep horizon the other placement with the present on the placement with the placement of the glycated and the FAOD is that the measured with a the placement of the glycated and the FAOD is that the measured with a the placement of the glycated and the faother and the glycated and the glycated and the faother and the glycated and glycat and the FAOD but also between the glycated emine and and the FAOD, so that the measured value of the glycated protein apparently increases. Mereover, regarding the above-described problem that some patients show considerable variation in the measured value depending on the time when the blood is collected even though the measurement is cerried out by the above-described method with respect to the whole blood samples collected from the same patient under the same conditions, the invertices of the procord invention size found out the following fact. Such as problem is seen mainly in patients after being put on an intravenous drip or the tixe. For example, if a seccharido such as glucose

and any of various amino acids are administrated via an intravenous drip or the like, a glycated amino acid is formed from such exogenous substances. As a result, the glycated amino acid increases temporarily, which ocurse the above-described variation in measured value. Thus, based on these findings, the inventors of the present invention discovered that, even if a whole blood sample contains a glycated amino acid present horizostatically or an exogenous glycated amino acid present temporarily, the increase in the measured value due to the glycated amino acid as described above can be suppressed by causing a degredation FAOD to set on the glycated amino acid to degrede it as in the present invention. This allows the accuracy of measurement to be improved, in addition, since this aftern blood to be collected any time regardies of whether or not the patients were put on an intravenous dry, the burden on patients can be reduced. Moreover, by carrying out the redox reaction in the presence of the tetrazolium compound and the sodium acide, the measurement accentivity is intervoved, although the mechanism is unknown. Therefore, according to the method that is excellent in measurement excursely and measurement analytic in the field of although the reliability of various glycated proteins as indicators is improved. Thus, the method is useful in the field of although medicine and the like.

[0011] In the present invention, the glycated protein preferably is glycated Hb. This is because the observed ascribed method of the present invention can improve the reliability of glycated Hb as an indicator in the diagnosis of disberge and thus can service as assist method in the field of clinical medicine and the like.

[0012] Examples of the method for measurement according to the present invention include a first method in which FAODs having substrate specificities different from each other are caused to act on the groated arrive edit and the glycated protein, respectively, and a second method in which FAODs having the same substrate specificity are caused to act on them.

[0013] As described later, there are various FAODs, e.g., a FAOD that acts on a glycated cremine group, a FAOD that acts on a glycated amine group in a side chain (hereinster give referred to as a "glycated alide-chain amine group) of an amine getal residue such as a lysine residue or an arginine residue, and a FAOD that acts on both a glycated amine group, and their substrate specificities vary depending on the type of FAODs. When the glycated protein is glycated the, for example, the amount of the glycated Ho can be measured by causing a FAOD to act on any of the glycated determine group, the glycated alde-chain amine group, and both the glycated describing group and the glycated side-chain amine group.

[0014] In the first mothod of the present evention, it is preferable that the degradation FAOD caused to act on the glycated arrive acid has a substrate specificity different from that of the measurement FAOD caused to act on the glycated protein. With this configuration, the glycated arrive acid is degraded with the degradation FAOD, and then, with regard to the glycated protein, the glycated is the thereof not subjected to the action of the degradation FAOD is subjected to the action of the measurement FAOD having a substrate specificity different from that of the degradation FAOD. Thus, the influence of the glycated arrive acid can be aliminated so that the accuracy of the measurement is

[0018] Specifically, it is proterable that the degradation FAOD is specific for a glycated c-amino group, and the measurement FAOD is specific for a glycated c-amino group and a glycated cide chain of an amino acid review, for example. Since the measurement FAOD sets on both a glycated co-amino group and a glycated side-chain emino group, it also exist on the glycated amino acid having a glycated co-amino group and a glycated side-chain emino group, it also stope. However, in the present invention, alno the glycated amino acid begraded with the degradation FAOD specific for glycated or emino group in advance, there is no chance that the measurement FAOD may set thereon. As a result, the secretary of the measurement is improved. Moreover, athough the measurement is improved. Moreover, athough the measurement FAOD acts on both a glycated cramino group and a glycated advantal eminor group as described above, since the glycated cramino group of the glycated protein also is degraded with the degradation FAOD, it is possible to cause the measurement FAOD to act only on the glycated side-chain amino group of the glycated protein. Therefore, this method particularly is useful for measurement of glycated Hb that is characterized by

the amount of the glycated alch-chain amine group.

[Outs] When using different FACDe as acceptibed above, it is preferable that the glycated protein is degraded with a preferable to glycated protein and the glycated protein and the glycated amine acid and the measurement FACD caused to act on the glycated amine acid and the measurement FACD caused to act on the glycated protein is to accept the set on this depreciation product as as to cause the above-described redox reaction. The degradation of the glycated protein with a proteins is carried out because FACDe have properties that they act on glycated amine acids and shorter phycated peptide fragments more vestly than on glycated proteins. Moreover, the reason why the proteinse traces where the store or after the degradation for glycated proteins and the glycated errine acid is that, since the measurement FACD also can act on the glycation site other than that on which the degradation FACD acts as described above, the

degradation FAOD treatment does not have any influence on the measurement of the glycated protein itself.

[0017] Next, as the second method of the present invention, it is preferable that the glycated protein is degraded with a protesse to give a degradation product of the glycated protein after causing the degradation FAOD to set on the glycated emino acid, and the above-described redox reaction is caused by adding the FAOD having the semie substrate.

specificity as the degradation FAOD so that it acts on the degradation product. That is, in the second method for measurement, the degradation FAOD and the measurement FAOD are the serve.

[Bd18] Specifically, it is preferable that the degradation FAOD is inactivated with the protease. As described above, FACUS have properties that they act on givested antino acids and shorter glycated peptide fragments more easily then on a grycated protein as an enalyte. Thus, it can be said based on chamical kinetics of enzymes that, even if a degradation FACUs acids, it hands a the acids and shorter glycated protein as an enalyte. Thus, it can be said based on chamical kinetics of enzymes that, even if a degradation FACUs acids, it hands acids an interpretable protein within a treatment period for degrading the glycated emine. ecid. However, if the activity acts of the degradation FACD additionable during the processe treatment of the glycated protein performed subsequently, the remeining degradation FAOD may act on a physicial protein degradation product (i.e., a performed succeptions, the remaining degreement FACU may act on a procure degreement representation process. It is, a givened amine seld and a glycated people fragment of the glycated protein) obtained while the glycated protein is being degreeded with the proteine. Therefore, when the measurement FACD is added after the proteines treatment, part of the glycated protein degreeded on product already is subjected to the ection of the remaining degreeded on FACD. As a result, contrary to what is intended, the occurroy of the measurement may be deteriorated. However, if the protesses treatment performed to degrade the glycated protein service to inactivate the remaining degradation FAOD at the service to inactivate the remaining degradation FAOD at the service time as described above, the glycated protein degradation product obtained by the protease (reatment remains unreacted with the degradeaton FACO and thus can make with the measurement FACO added subsequently. As a result, the accuracy of the measurement can be improved.

the securacy of the measurement can be improved.

[D019] On the other hand, as a libit direthoof for measurement, highly accurate measurement also can be realized by, for example, adjusting the amounts of the degradation FAOD and the measurement FAOD to be added to the sample without fractivating the degradation FAOD by the protesse treatment as described above, in this case, the earners without sectionality of the degradation FACD (A) to the mossurement FACD (B) preferably is set in the range from 1:10 to 1:1000. When the ratio is in the above-described range, if the degradation FACD remains during the professes freatment, the remaining degradation FAOD hardly acts on a glyculard protein degradation product, as understood from the chemical kinetics of enzymos,

(0020) In the mothod for massurement according to the present invention, as the protesse, at least one protesse selected from metalloproteinasses, brometels, papein, trypsin, proteinase K, subdistin, eminopoptidase, and protesses

(0021) When the glycated protein is glycated Hb, the protease preferably is at least one protesse that degrades the glycated His selectively and is selected from the group consisting of metalloproteinases, bromelain, papain, typein derived from porcine pancross, and protease derived from Bacifius subtiles. Among those, metalloproteinases and protesse derived from Bacilius subtilis are proferable, and metalloproteinesses are more preferable. When the enalyte is glycated Hb, the givested Hb skine can be measured by using such a probase, because givested profeins and glycated populates other than the glycated Hb hardly are degraded with the protease and thus a FACD hardly acts on the glycated proteins and the like other than the glycated Hb.

[0022] In the method of the present invention, it is preferable that the terrecollum compound (C) and the sodium Azide (D) are present at a ratio (molar ratio C : D) in the range from 20 : 3 to 20 : 12. Furthermore, it is preferable that a final concentration of the tetrazolium compound in a reaction solution of the redox reaction is in the range from 0.5 to 2.5 mmoVI, and a final concernation of the audium azide in the reaction solution is in the range from 0.13 to 1.3 mmoV. [0023] In the method for measurement according to the present invention, it is presented that a solution containing the tetrazolium compound and the sodium azide is agod and then is added to the sample because this allows add further improvement in the sensitivity. In this case, it is proferable that the solution is aged at a temperature in the range form 20°C to 90°C. Furthermore, it is preferable that the solution is aged for 6 to 120 hours.

[0024] In the present invention, the measurement of the rodox reaction is not perfectively limited, and may be, for

(0024) In the present invertion, the measurement of the robox reaction is not periodizely tended, whe may up, as example, measurement of an amount of hydrogen periodic formed by causing a redox reaction between the hydrogen periodic is determined by causing a redox reaction between the hydrogen periodic end a substrate that develops color by exidetion (color-developing substrate) and then measuring the amount of the color developing substrate. As the color-developing substrate, N-(carboxymathyteminocarbopy) 4.4-bis (directly/amino)cliphenylamine codium anti (bereinstor elso reterred to as "DA-64") can be used, for example, [0025] When DA-64 is used, it is preferable that the DA-64 is godded to the reaction solution in the presence of a surfactam. Also, it is protestable that the concentration of the terracelum compound in the reaction solution is in the range from 0.5 to 8 mmoVI, the concentration of the sodium abide is in the range from 0.08 to 0.8 mmoVI, the concentration of the sodium abide is in the range from 0.08 to 0.8 mmoVI, the concentration of the sodium abide is in the range from 0.08 to 0.8 mmoVI, the concentration of the sodium abide is in the range from 0.08 to 0.8 mmoVI. tration of the auriectant is in the range from 0.3 to 10 mmoVI, and a pH of the reaction solution is in the range from 7.0

[0028] It is one of the features of the present invention that the redox reaction is caused in the presence of a telescalum compound and sodium aside in order to improve the measurement sensitivity. However, when DA-64 is used zotum compound and sociatin acros in ottor to emprove the mossulement sensitivity. However, when LAPPA is used as the color-developing substrate, a color development error of the DA-84 may occur due to the presence of the telescolum compound and acadium azide with the DA-84. Such color development error leads to an increase in background in the measurement of the color developed and to a shortage of the DA-84 even though the amount of the DA-84 added is entitledent. However, when the DA-84 is added in the presence of the surfactant and the concentrations of the re-

specifive components and the pH of the reaction activition are sat in the above-described ranges, the increase in background can be suppressed so that the measurement can be carried out still more securately.

ground den us suppressed so trat tre measurement can be carried out six more excursion.

[9027] In the method for measurement according to present invention, the type of the sample is not particularly limited. The method also can be applied to samples other than whole blood, pleams, serum, and blood calls, e.g. biological samples such as surface and worresterning to the samples such as surface and worresterning. sauce. Among these, the mathod particularly is useful for a whole blood sample and a blood cell sample. For example, when glycetted His in erythrocytes is to be micasured, whole blood light may be homolyzed to prepare a sample, or orythrocytes may be expensived from whole blood and hemolyzed to propers a sample.

[0028] Examples of the glycated protein as the analyse include glycated Hb as described above and glycated abounts.

[0028] Examples of the glycated protein Among these, glycated Hb is proferable.

Management, gyester must preventible.

[10029] Next, the present invention provides a method of determining a ratio of glycated Hb to Hb. The mothod includes: measuring an amount of glycated Hb in a sample by the share-described method of measuring glycated Hb. according to the present invention; measuring an amount of Hb in the sample; and then calculating the ratio of the glycated Hb and the amount of the Hb this measured. According to the present invention, the amount of the givested Hb can be measured with high accuracy and hence, a highly reliable value of the ratio of the glycated Ho can be obtained. It is to be noted here that the "amount of Hib" refers to the total amount including both the amount of gheated Hb and the amount of non-givented Hb.

[00:00] In the present invention, the method of measuring the amount of Hb is not particularly limited, but preferably is a method including: denaturing Hb in a sample with a totrazellum compound to give denatured Hb; messuring an absorbance of the sample at an absorption wavelength specific to the denatured Hb; and calculating an amount of the Hib in the sample from this absorbance. The Hib that is not yet denatured (hereinative, referred to as "undenatured Hib") where it is not bound to oxygen, stc. Therefore, it is difficult to determine the amount of Mb accurately by morely measuring the absorbance. In contrast, the donatured Hb obtained by the treatment with the tatrazofum compound is stable and exhibits the absorption maximum at a wavelength falling within a certain range. Thus, according to the method of the present invention, the amount of Hb can be measured easily and accurately. Therefore, by using the above-described highly reliable values of the amount of the glycated Hb and the amount of the Hb, 8 still more reliable value of the ratio of the glycated Hb to the Hb can be obtained.

[DG31] The method of measuring a glycated protein according to the precent invention also can be used to measure NbA1c. The amount of HbA1c in a sample can be determined in the following manner, for example. First, a calibration curve is prepared based on a correlation between an amount of glyceted Hb obtidned by the mathod of measuring a glycated protein according to the present invention and an amount of HbA1o. The arount of phycated Hb in a semple is massured by the method of the present invention and the obtained measured value is substituted into the calibration curve to determine an amount of HbA1c in the eample.

[0092] The inventors of the present invention have conducted in-depth researches and finally found that there is a The arranges of the present grounds may consume the consumer of the present of the method of measuring an amount of glycated Hb in whole blood obtained by the method of measuring an amount of glycated Hb according to the present invention and an amount of HbA1c in the same expeller. HbA1c is glycated Hb in which the N-terminal α-amino group in the β-chain of Hb is glycated. In glycated Hb, HbA1c serves as a particularly important indicator in the diagnosis etc. of diabetes. According to conventional methods of measuring HbA1c, it is necessary to cause a FAOD to act specifically on the plycated N-terminal α-emine group in the β-chain as a plycation site characteristic to HbA1c and then to measure the rodox residion caused by the FAOD. To this end, special techniques are required because it is necessary that the FAOD used has a high substrate specificity to the giyested oamino group and that the FACD acts on the glycated elembro group sufficiently, for example, in contrast, according to the precent invention, the amount of HbA1c can be determined based on the amount of glycated the measured with high occursoy. This enables accurate and seay measurement of HbA1c. As a result, the measurement of HbA1c can be made practical in clinical tests etc.

[0033] Next, the present invention provides a measuring kit used for measuring a glycated protein using a redox reaction, including: a pretreatment reagent for pretreating a comple, containing a FAOD; and a color-developing magent containing a FAOD, an oxidoreductree, and a color-developing substrate.

[0.34] As described above, the inventors of the present invention found out the cause of the problem regarding the measurement securesy. Then, the inventors of the present invention discovered that, even though a whole blood wample contains a glycetod emino acid present homeostatically or an exogenous glycatod emino acid present temporarily, if a FAOO is contained in the profrestment reagent as in the present homeostatically or an exogenous glycatod emino acid present temporarily, if a FAOO is contained in the profrestment reagent as in the present invention, the glycated emino acid in the sample to degraded by adding the prefreatment reagent to the sample prior to the redox reaction and thus the increase in the magaured value due to the glycated amino acid as described above can be suppressed. Therefore, by using a massuring bit according to the prepent invention, the measurement of a glycated protein using a redox reaction can be carried out quickly and aimply and besides, with high accuracy. Moreover, with respect to a blood sample obtained from a patient after being put on an intravenous drip, the measurement can be carried out under the same conditions.

Furthermore, since the measuring kit according to the present invention can carry out measurement with high accuracy as described above, it can increase the reliability of vertous giyosted proteins as indicators and thus can serve as a structure in the measuring kit in the field of circuit medicine and the like, there is no the measuring the contract of the contract of the measuring kit according to the contract of the contrac

[0025] In the measuring kit according to the present invention, it is preferable that an enalyte is glycated Hb. This is because the reliability of glycated Hb as an indicator in the diagnosts of diabetre is increased by this measuring kit so that glycated Hb becomes more useful in the floid of clinical medicine and the like.

[0025] In the present invention, a FACO contained in the profressment reagent degrades the glycated amino acid.

Thus, such a FAOD hereinalize rate to sa a "degradation FAOD" as described above. On the other hand, a FAOD contained in the color-developing magent is caused to act on a givested protein, and thus hereinalize also is referred to as a "neasurement FAOD".

(0037) It is proferable that the measuring kit according to the present invention further includes a professe respent containing a professe. FAODs have proportice that they set on glycated amino acide and aborter glycated poptide fragments more easily then on glycated professe. Therefore, by degrading a glycated profess in a sample by this professe, the measurement FAOD can act on the glycated professes are used in the mather improved. As the protesses, the same professes are used in the method of measuring a glycated profess are used in the method of measuring a glycated professes.

eccuracy to be rurater improved. As the protested, the same protested as used in the method or measuring a givested protein according to the present invention, at its preferable that the protested magent further contains a tetrazolium compound and addum azide. When the protested reagent contains a tetrazolium compound and sodium azide, when the protested reagent contains a tetrazolium compound and sodium azide, the influence of reducing substances and the like contained in the sample on a redox reaction can be oliminated by the tetrazolium compound, thereby eligibling the measurement accuracy to be improved.

[8039] When the protease contained in the protease reagent is a metallioproteinase, it is preferable that the protease reagent further contains Ca and Na. Preferably, the concentration of the metallioproteinase is in the range from 100 to 40,000 KU/I, the concentration of Ca is in the range from 0.1 to 50 mmol/I, and the concentration of Na in in the range from 6 to 1000 mmol/I. When the metallioproteinase, Ca, and Na ere present in the protease reagent so that their concentrations fall in the above-described ranges, the stability of the metallioproteinase is improved. As a result, not only at low temperatures but size at ordinary temperatures, the metallioproteinase is prevented from being hysothetical and thus can be stored stably, for example. The lonization of Ca and Na to Ca²⁺ and Na*, respectively, may occur during the use of the preferee reagent.

during the ura of the professe reagont.

[2040] The concentrations of the respective components in the protease magnetian not limited to the above-described ranges. For example, it is preferable that the ratio between the respective components is the same as that between the respective components in the same as that between the respective components mocessay for the reaction can be adjusted by adjusting the proportion of the protease reagent to the reaction solution (i. e., offution ratio). The same applies to other components and other registrate.

Examples of the measuring kit according to the present invention include a first measuring Rk in which a degradation FAOD contained in the profrestment reagent and a measurement FAOD contained in the color-developing reagent have substrate specificities different from each other, and second and third measuring kits in which the degradation FAOD and the measurement FAOD have the same substrate specificity.

[0042] In the first measuring kit according to the present invention, it is preferable that the degradation FAOD contained in the pretreatment reagent has a substrate specificity different from that of the measurement FAOD contained in the color-daveloping reagent. When the measuring kit having such a configuration is used, the glycated amino acid is degraded with the degradation FAOD, and then, with regard to the glycated proton, the glycation sits thereof not subjected to the action of the degradation FAOD is subjected to the action of the measurement FAOD. Thus, the influence of the glycated amino acid can be eliminated as that the accuracy of the measurement is improved.

[0043] Specifically, it is preferable that the degradation FAOD is specific for a glycated a-amino group, and the measurement.

Specifically, it is preferable that the degradation FAOD is specific for n glycated dramino group, and the measurement FAOD is specific for a glycated dramino group and a glycated dramino article group, for example. Since the measurement FAOD acts on both a glycated dramino group and a glycated dischain amino group, it also acts on the glycated arrive according a glycated arrive the glycated arrive according to the present invention is used, since the glycated amino acid is degraded with the degradation FAOD specific for a glycated cramino group in advance by the attendment when it is no chance that the measurement FAOD may act thereon. As a result, the seeming increase in the measurement FAOD acts on both a glycated cramino group as developed elide-chain entire group as described above, the glycated cramino group of the glycated elide-chain entire group as described above, the glycated cramino group of the glycated selection for the glycated cramino group as described above, the glycated cramino group of the glycated selection for the glycated gramino group of the glycated selection for the glycated protein. The sample is trended with the color-developing negant lacedrifting to the present invention, the measurement FAOD can set only on the glycated did-chain amino group of the glycated protein. The measurement FAOD can set only on the glycated elid-chain amino group of the glycated protein. The measurement fAOD can set only on the glycated elid-chain amino group of the glycated protein. The measurement fAOD can set only on the glycated elid-chain amino group of the glycated protein. The measurement fAOD can set only on the glycated elid-chain amino group of the glycated protein. The measurement fAOD can set only on the glycated elid-chain amino group of the glycated protein. The measurement fAOD can set only on the glycated elid-chain amino group of the glycated protein.

group. Such a manacuring kit can be used in the first method of measuring a glycated protein according to the present

Invention.

[3044] In the second measuring kit according to the present invention, the degradation FACD contained in the protreatment reagent and the measurement FAOD contained in the color-developing reagent have the same substrate specificity. Preferably, the second measuring kit further includes a protesse rangent containing a protesse for degrading a glycated protein and inactivating the degradation FACO by digesting it.

a glycated protein and inactivating the degradation FACO by digasting it.

[BB45] The second measuring lit can be used in the following manner, for example. First, the pretreatment reagons is added to a sample so that the degradation FACO exts on a glycated amino acid. Therefore, the protesse reagons is added so that the operation degradation fACO extra degradation protein and, at the same time, degradate the remaining degradation FACO by digasting it. Then, the color-developing reagent is added so that the measurement FACO acts on the plycated protein degradation product obtained by the proteins treatment to cause the above-decenters meeting.

(0046) As described above, FAODs have properties that they act on glycated amino scide and shorter glycated peptide hagments more easily than on a glycated protein as the analyte. Thus, it can be said based on chamical thretica of enzymes that, even though the pretreatment respent containing the degradation FAOD is added, the degradation FAOD hardly acts on the glycated protein within a treatment period for degradating the glycated armino acid. However, if the activity of the degradetion FAOD still remains during the treatment with the protosse reagont performed subsequently, the remelating degradation FAOD acts on a giyested protein degradation product (i.e., a giyested emino acid and a glycated peptide fragment of the glycated protein) obtained while the glycated protein is being degraded with and a glycardo propule fragment of the glycardo protein obtained winks una glycardo protein is being segrected with the protease. Therefore, when the color-developing magnetic containing the measurement FAOD is edded after the treatment with the protease reagent, part of the glycardo protein degradation product dready is subjected to the action of the degradation FAOD. As a result, contrary to what is intended, the accuracy of the measurement may be determined. crated. However, if the protesse reagent that serves to degrade the glycated protein and inactivate the degradation FAOD by diposting it is further included as in the second measuring kit according to the present invention, the phycated protein agradation product remains unreacted with the degradation FAOD and thus can make with the measurement FACD in the color-developing respont added subsequently. As a result, the accuracy of the measurement is improved. Such a measuring kit can be used in the second method of measuring a givented protein according to the present

[0047] In the third measuring kit according to the present invention, the degradation FAOD and the measurement FAOD also have the same substitute specificity, Howaver, the third measuring kit can restize highly accurate measurement without in activating the degradation FAOD with a protesse respont as in the second measuring kirby, for example, adjusting the concentration of the degradation FAOD in the protestment reagent and the concentration of the measuring the concentration of the concentration of the measuring the concentration of the measuring the concentration of the concentration grament FAOD in the color-developing reagent, in this case, the concentrations of the degradation FAOD in the pre-treatment reagent and the measurement FAOD in the color-developing reagent preferably are set so that the ratio rections reagons and the measurement of the measurement FACO (E) in, for example, a color-developing reaction solution obtained finally fails in the range from 1 : 10 to 1 : 1000. When the ratio is in the proved-described range, even if the degradation FACO remains during the treatment with the proteste reagons, the remaining degradation FACO hardly acts on a ghosted protein, se understood from the chemical kinetics of enzymes. Such a measuring kit can be used in the third method of measuring a glycated protein according to the present invention.

[0045] In the massuring kit according to the present invention, as the color-developing substitute, a substitute that develope color by existing a substitute that develope color by existing a substitute that develope color by reduction, or the like can be used, for example. Among these, a substrate that develops color by oxidization is preferable, and specifically. N-(carboxymethyleminocarbonyl) 4,4"-bis (dimethylamino) diphonylamino zodium seit is preferable,

In the case whore the DA-84 is used as the color-developing authorate, when the protease reagant contains in terractions compound and sodium ables as described above, these three components are mixed with each other in the reaction system, which may cause a color development error of the DA-64. Such a color development error leads to an increase in background is the monaurement of the color developed and to a shortage of the DA-54 even though to an amount of the DA-84 added is sufficient. However, as described above, if the respective components are contained in the respective reagons so that their concentrations fall within the above-describe ranges, the other development error of the DA-94 in the reaction system is suppressed. As a result, the increase in background can be suppressed so that the management can be carried out still more accurately.

as that the measurement can be carmed our aski more securatory.

[DUSO] In the freesouring kit according to the present invention, each of the pretresoment respent, the protesse reagent, and the color-developing respent turther may contain a surfactant.

[DUSO] Furthermore, it is preferable that each of the resignite further contains at least one buffer selected from the
group consisting of CHES, MOPS, MES, Tile, phosphate, TES, TAPS, HEPESO, MEPPSO, borets, methanolamine. BES, MOPSO, EPPS, POPSO, ADA, PIPEB, ACES, and Bis-Tria. Each of the respents contains an entyrne. Thus, by edding the buffer, it is possible so cause the anzyme to act at an optimal ph, for example, and an enzyme, and the control of the profession of the profe

preirealment reagent is in the range from 8.0 to 10.0.

(0063) Furthermore, it is preferable that the protesse reggent further contains at least one buffer selected from the group constaining of Tris, MES, CIPSO, TES, POPSO, HEPES, MOPSO, Bis-Tris, MOPS, AOA, PIPES, ACES, and phosphate, and that a pH of the protesse rangent is in the range from 5.0 to 7.0.

[2084] Still further, it is preferable that the color-developing respent further contains at least one buffer extected from the group consisting of MES, This, phosphete, MOPS, TES, HEPES, HEPESO, and EPPS, and that a pH of the colordeveloping reagent is in the range from 0.0 to 8.0.

[0065] Preferably, the color-developing rangent further contains addium axide because it can prevent the color development error of the developing substrate such as OA-84, for example.

[0066] Preferably, the protreament reagon! further contains at least one of urbase and billrubin axidase. When the profreement reagont contains uricase, uric acid contained in a sample can be degraded, and when the pretreatment rangent contains bilitable exidese, bilitable in a semple can be degraded, for example. The unit add and bilitable have reducing power. Thus, by degrading the unclassed and bilitubin as described above, the influence of the reducing aubdanged can further be eliminated, which allows the accuracy of measurement to further be improved.

[0057] Examples of the specific composition of each of the reagents in the measuring && eccording to the present

Invention will be operating before the factor of the FAOD is aspectic for a glycated e-amino group and that the concentration of the FAOD is in the range from 10 to 5000 U/I and the concentration of the FAOD is in the range from 10 to 5000 U/I and the concentration of the butter is in the range from 5 to 200 mmol/i. Furthermore, a pH of the preferational reagont preferably is in the range from 8.0

[0059] For example, in the protesse reagent, it is pretarable that the concentration of the metalloproteiness is in the range from 100 to 10,000 KU/s, the concentration of the tetracellum compound is in the range from 0.1 to 10 mmol/s, the concentration of the sodium azido is in the range from 0.8 to 4 mmol/s, the concentration of Ca is in the range from 0.1 to 50 mmoVI, the concentration of Na ta in the henge from 5 to 1000 mmoVI, and the concentration of the buffer b in the range from 0.1 to 500 mmot/l, Furthermore, a pH of the protease reagont preferably is in the range from 5.0

(20160) For example, in the color-developing reagent, it is preferable that the FAOD is specific for a glycated examino group and a glycated side chain of an amino acid residue and that the concentration of the FAOD is in the range from 100 to 50,000 U/I, the concentration of a peroxidace is in the range from 0.1 to 400 KU/I, the concentration of N-(can-boxymethylaminocarbonyl)-4,4'-bis(directrylamino)dipherrylamine sett to in the range from 0.02 to 2 mmol/I, and the concentration of the buffer is in the range from 10 to 500 most, Furthermore, a pM of the cotor-developing reagoni preferably is in the range from 6 to 9.

[2001] The measuring lift eccording to the present invention is applicable to the same samples as in the method of measuring a glycated protein according to the present invention. Also, the measuring kit is applicable to the come analytics as in the method of measuring a givested protein according to the present invention, and givested Hb is referable as an enelyte.

[0062] Each of the responts in the measuring kit occording to the present invention may be a liquid reagent obtained by despiving respective components in a squeous servent or a dry respent to be dissolved in an equeous servent

Brief Description of Drawings

[0063] FIG. 1 is a graph showing the correlation between the amount of HbA1c measured by an enzymatic method according to one example of the present invention and that measured by HPLC.

Bord Mode for Carrying Out the Invention

[0064] In the method for measurement and the measuring kit according to the present invention, FACIDs estallyzing a reaction represented by Formula (1) below preferably are used. Examples of such FAODs include a FAOD specific a reaction represented by Formula (1) below preferably site used, exemples or such FACUs increde a FACU specific for a glycated emine having a glycated c-amino group (hereinafter referred to as a "FACU-or"), a FACU specific for a glycated amine having a glycated smine having a glycated smine having a glycated smine group in a side chain of an amino stude (hereinafter referred to as a "FACU-S"), and a FACU specific for both a glycated protein having a glycated a-amino group and a glycated protein having a glycated amino group in a side of an amino acid residue (horokraftor referred to as a "RADD-cx5"). The "plycated amino" refers to a glycated protein, glycated popide, glycated amino acid, and the like.

H'-CO-CH,-NH-H2 + H,O + O,

sented by Formula (1) in this case,

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$$\rightarrow \text{R}^{\dagger}\text{-CO-CHO} + \text{NH}_2 \cdot \text{R}^2 + \text{H}_2\text{O}_2 \tag{1}$$

[0085] In Formuta (1), Fi[†] denotes a hydroxyl group or a residue derived from the sugar before glycation (i.e., sugar residue), The engar residue of the sugar before glycation is addess, and is a kekese residue when the sugar before glycation is didess, and is a kekese residue when the sugar before glycation is glucose, it takes a fructose structure effer glycation by an Amedori reamongement, in this case, the sugar residue (Fi[†]) becomes a glucose residue (an aldose residue). This sugar residue (Ri[†]) can be represented, for example, by

-{CH(OH))_n-CH₂OH

where n is an integer of 0 to 6.
[0066] In Formula (1), R² is not particularly limited. However, when the glycated emine is a glycated emine exid or agreement populate (including a glycated protein), there is a difference between the case where an at-amino group is glycated and the case where an at-amino group is glycated and the case where an amino group other than the at-amino group (1.e., an amino group is a side chain of an

amino acid residue) is glycated.

[0087] In Formula (1), when an a-amino group is glycated, R² is an amino acid residue or a peptide residue represented by Formula (2) below. The above described FAOD-a and FAOD-aS specifically catalyze the reaction repre-

65 [00:68] In Formule (2), R² denotes an amino-acid aido chain group. R⁴ denotes a hydroxyl group, an amino-acid nealdue, or a popide residue, and can be represented, for example, by Formula (3) below. In Formula (3), n is an integer of 0 or more, and R² denotes an amino-acid aido chain group as in the above. When n is an integer of more than 1, the amino-acid aido chain groups may be either the same or different.

[0069] In Formula (1), when an amino group other than the α-amino group is glyceted (i.e., an amino-acid side chain group is glyceted). P2 can be represented by Formula (4) below. The above-described FAOD-9 and FAOD-9S specifically catalyze the reaction represented by Formula (1) in this case.

[0070] In Formula (4), R³ denotes a portion other than the glycated amino group in the emino-acid side chain group. For example, when the glycated amino acid to tysine, R³ is as follows.

For another example, when the phycated amino sold is arginine. At is as follows.

+CH2+CH2+CH2+NH-CH(NH2)

[0071] In Formula (4), R^e denotes hydrogen, an amino acid residue, or a poptido residue, and can be represented, for example, by Formula (5) below, in Formula (5), a denotes an integer of 0 or more, and R³ denotes an amino-acid side chain group as in the above. When n is an integer of more than 1, the amino-acid side chain groups may be either the same of different.

[0072] In Formula (4), R² denotes a hydroxyl group, an amino acid residue, or a peptide residue, and can be represented, for example, by Formula (6) below. In Formula (5), n is an integor of 0 or more, and R² denotes an amino-acid also chain group as in the above. When n is an integer of more than 1, the amino-acid side chain groups may be either the same or different.

-(NH-CHR³-CO)_n-OR

[0073] Exemples of the FAOD-a specific for a glycated a-entitio group include a commercially available product named Fructosyl-Amino Acid Oxidese (FAOX-E) (manufactured by Kikkoman Corporation) and FAODs derived from the genus Fancillum (LP 8(1985)-398388 A). Examples of the FAOD-S specific for a glycated side chain of an arrino acid residue include FAODs derived from the genus Fusarium ("Conversion of Substrate Specificity of Amino Acid Oxidese Derived from Fusarium anyaporum" by Maid FUJWARA at al., Annual Meeting 2000, The Sodoty for Biotechnology, Japan). Furthermore, examples of FAOD-aG specific for both a glycated d-emino group and a glycated side chain group of an arrino acid residue include a commercially available product manual FOO (manufactured by Acaid Chemical Industry Co., U.d.), FAODs derived from the genus Fusarium (LP 7(1985)-389253 A), and FAODs derived from the genus Appendix (WO 88/20039).

[0074] The fotrecollum compound used in the present invontion preferably contains ring substitutions at least at two

positions on its terrarole ring, more protectely at three positions on its tetrarole ring, for example.

[0075] In the case where the tetrarollern compound comains ring substituents at least at two positions on its tetrarole ring as described above, it is preferable that the ring substituents are at the 2-position and 3-position on the tetrarole ring. Further, in the case where the tetrarollern compound comains ring substituents at three positions on the tetrarole ring, it is preferable that the ring substituents at the 2-position, and 6-position on the tetrarole ring.

[0076] Further, it is preferable that at least two ring substituents of the tetrazolium compound have a benzano ring structure. Basidos the benzane ring structure, the ring structure with S or O being contained in the ring skieteron, for example. Examples of the ring substituents with such a resonance structure include a thloryl group, thistoryl group, and the time.

[0077] Furthermore, it is preferable that the tetracollum compound contains ring substituents at least at three positions on its tetracole ring and at least two of the ring substituents have a benzene ring structure.

(0078) Still further, it is preferable that at least one ring substituent contains a functional group, and a larger number of functional groups are more preferable.

(0079) As the functional group, an electron-withdrawing functional group preferably is used. For example, a halogon group, ether group, ester group, earboxy group, expl group, nitrose group, hydroxy group, suffer group, earboxy group, expl group, nitrose group, hydroxy group, suffer group, and the like; and characteristic groups express exchanging using explore, pooling group, box group, and the like; and characteristic groups contribing using group, explored as a mercante group, allytible group, methyliklemethyl group, there group, suffered group, benzenesulteryl group, phonyselforly group, p-totylaufonyl group, totyl group, suffered group, a nitro group, and the like also can be used, for example, Among these electron-withdrawing functional groups, a nitro group, autic group, halogen group, carboxy group, hydroxy group, methoxy group, schoxy group are preferable. Further, in addition to the above-described electron-withdrawing functional groups, unsaturated hydrocarbon groups such as a phenyl group (C₆H₂-), stryr) group (C₆H₂-), and the like also can be used, for example, it is to be noted that the functional groups.

Stif further, it is preferable that the terrezolium compound contains benzone rings at the 2-position and 3-po-

[0090] Still further, it is preferable that the terriscollum compound contains benzone rings at the 2-position and 3-position on the tetrazole ring and at load one of the benzone rings contains at least one functional group selected from the group consisting of a halogan group, carboxy group, hitro group, hydroxy group, suito group, methoxy group, and ethoxy group. It is to be noted here that both the benzene rings may have such a functional group. Further, the functional group may be contained at any positions (ordino, mete-, pare-) on each of the benzene rings. Furthermore, the number of the functional groups is not particularly limited, and the benzene ring may have either the series or different functional groups.

(0081) Examples of the tatrazoflum compound containing ring substituents having a benzene ring structure at the 2-position, 3-position, and 5-position on its tetrazofs ring include:

- 2-(4-ladophenyi)-3-(4-nibophenyi)-5-(2,4-disultaphenyi)-2+-tatrazolium sait (hereinaltar also referred to as "WST-
- 27;
- 2-(4-lodophonyl)-3-(2,4-dinitrophonyl)-5-(2,4-disulfophonyl)-2H-tetrazolium calt;
- 2-(2-mattaxy-4-nitrophenyi)-8-(4-nitrophenyi)-5-(2,4-disuftophenyi)-2H-totrazolium snii;
- 2-(4-lodophenyi)-3-(4-nitrophenyi)-5-phanyi-2H-retrazollum selt:

- 3,3'-(1,1'-biphenyi-4,4'-diyi)-bis(2,5-diphenyi)- 2H-tetrazolium sali;
- 3.3"-[3,3"-dimethoxy-(1,1"-biphenyl)-4.4"-diyl)-bis[2-(4-nitrophenyl)-5-phonyl-2 H-tetrazolium call);
- 2,3-diphonyl-5-(4-chlorophonyl) tetrezolium solt:
- 2,5-diphonyl-3-(p-diphenyl) totrozolium selt:
- 2,3-diphenyi-3-(p-diphenyi) tetrazolium sak; 2,5-diphenyi-3-(4-styryiphenyi) tetrazolium sait;
- 2,5-diphenyl-3-(m-tolyl) tetrazolium salt; and
- 2,5-diphenyl-9-(p-tolyl) tetrazolium salt.
- [0082] The tetracollum compound is not limited to those described above, in addition to the above-described tetrazollum compounds, a tetrazollum compound containing ring autotituente having a benzone ring structure at two positions and a ring substituent having a ciructure other than the banzone ring structure at one position on its tetrazole ring also may be used. Exemples of such a tetrazolium compound include:
- 2.3-diphonyl-5-(2-thlenyl) tetrazolium salt;

 - 2-benzothlazoyl-3-(4-cs/boxy-2-mathoxyphonyl)-5-(4-(2-sulfosthyl carbamoyl) phenyl]-24-tetrazolium exit; 2,3'-dibenzothlazoyl-5,5'-bia [4-di(2-sulfosthyl)carbamoylphenyl]-3,3'-dimothoxy-4,4'-biphenylene)dictrazo-
 - 3-(4,5-dkmethyl-2-thiszoyi)-2,6-diphonyl-2H-retrazolium anti.

[0083] Further, a tetrazolium compound containing ring substituents having a benzene ring structure at two positions and A substituent not having a ring structure at one position on its terrezale ring size can be used. Exemples of such a tetrazolium compound include:

- 2,3-diphenyl-6-cyano totrazolium selt:
 - 2,3-diphenyl-5-carboxy tetravollum sett;
 - 2,3-diphenyl-5-mothyltotrazollum selt; and
 - 2.9-diphenyl-5-ethyl totrazolium sall,
- [0084] Among the above-described tetrezoilum compounds, the tetrezoilum compounds containing three ring autsituants are preferable as described above. Among there, the tetrazollum compounds containing three this substitu-ents having a banzans this structure and a large number of electron-withdrawing functional groups are more professible. and WST-3 is particularly preferable. It is to be noted here that the above-described tetrazolium compounds may be a selt or may have been lonized, for example. Moreover, the telrasolium compound may be used either stone or in combinations of two or more types.
 - [0085] Hareinafter, the method of measuring a giycated protein according to the present invention will be described in detail with reference to the following Embodiments A-1 to A-S, in which glycated Ho in blood cells is measured.

(Embodiment A-1)

- [DOSS] The present embodiment is an example where a FACO-x is used to degrade a glycated amine acid while a FAOD-aS is used to measure phreated Hb and a redox reaction is caused in the presence of a tetrazolium compound
- [0067] First, whole blood likeli in hemolyzed, or a blood cell fraction is separated from whole blood in the usual way such as centrifugation and then hamolyzed, as as to prepare a hamolyzed sample. The method of crueing the hamolysis is not perficularly limited, and can be, for example, a method using a surfactant, a method using utrasante waves, and a method utilizing a difference in carnotic pressure. Among these, the method using a surfactant is prefemble because of its simplicity in operation, etc.
- [DOSA] As the surfactant, for example, non-tonic surfactants such se polyoxyethylene-p-t-actylphonyl other (e.g. Triton series surfactants), polyoxysthylene sorbitan nikyl exter (e.g. Tween series surfactants), polyoxysthylene alkyl ether (e.g. Brij series surfactants), and the tike can be used. Specific examples are Triton X-100, Twoon-20, Brij 35. and the like. The conditions of the treatment with the surfactant usually are as follows: when the concentration of blood cells in the solution to be treated is in the range from 1 to 10 vol%, the surfactant is added so that its concentration in the solution falls in the range from 0.01 to 5 with, and atimed at room temperature for about several seconds (about 5

 - [0089] Nart, a tetrazolium compound and sodium azide are added to the hemolyzed sample.
 [0090] When the compound and blood cells in the solution to be treated to in the range from 0.2 to 2 yells, the tetrazollum compound proforably is added so that its concentration in the solution falls in the range from 0.005 to 400

mmot/l, more preferably from 0.02 to 100 mmot/l, and particularly protonably from 0.1 to 50 mmot/l. Specifically, when the tetracellum compound is WST-3, it preferably is added so that its concentration fells in the range from 0.004 to 16 mmot/l, more preferably from 0.1 to 5 mmot/l. The tatracellum compound may be used either alone or in combinations of two or more types. By adding the tetracellum compound and addien acide as described above, the sensitivity becomes about 1.2 to 3 times greater than in the case where they are not added.

[0091] Furthermore, the tetrezoitum compound (C) and the sodium exide (D) are added so that they are present at a ratio (motor ratio C: D), for example, in the range from 20: 3 to 20: 12, proferably from 20: 6 to 20: 11, and more preferably from 20: 6 to 20: 10.

- [0992] The tetrazollum compound and addism azide may be added to the hemolyzed sample aimply set they are. However, in terms of simplicity in operation etc., it is preferable to use a tetrazollum compound solution obtained by dissolving the tetrazollum compound in a solvent and a codism azide solution obtained by dissolving the codism exide in a solvent, or a liquid modure containing both the tetrazollum compound and the acidium azide liquid modure containing both the tetrazollum compound and the acidium azide liquid modure.
- 18 [0.051] The concentration of the terrezollum compound or the sodium axide in the above-described respective solutions can be determined as appropriate depending on the dilating factor of the solutions when they are added to the hemolyxed sample, etc., but the concentration of the tetrezoltum compound is, for example, in the range from 0.1 to 10 mmonly, proferably from 0.6 to 5 mmonly, and more preferably from 0.7 to 2.7 mmonly, and the concentration of the application of the concentration of the concentra
- 20 : 5 to 20 : 11. and more preferably from 20 : 8 to 20 : 10.

 [D034] As the solvent of the above-described solutions, MOPS, MES, MOPSO, DIPSO, TES, POPSO, HEPES, phosphate buffer solutions, and the like can be used, for example. Among these, MOPS and MES buffer solutions are preferable. The pri of the solvent is, for example, in the range from 5.0 to 7.0, preferably from 5.5 to 6.6. The concentration of the buffer solution is, for example, in the range from 0.1 to 10 mmol/l, preferably from 1 to 5 mmol/l. The final concentration of the buffer solution after being added to the hemolyced example is, for example, in the range from 0.7 to 8 mmol/l, preferably from 0.8 to 4.8 mmol/l, and more preferably from 0.8 to
- Moreover, the tetrazellum compound-sodium azide liquid mixture prepared preferably is left for a certain period before being added to the hemotyzed sample so as to be aged, because this allows still further improvement in assettivity. According to this eging treatment, the assettivity becomes, for example, about 1.2 to 8 times greater than in the case where the aging treatment is not performed.
- the case where the aging treatment is not performed.

 [0098] In the eging treatment, the treatment temperature preferably is in the range from 40°C to 60°C, more preferably from 50°C to 60°, and the treatment period is, for example, in the range from 6 to 72 hours, preferably from 15 to 20 hours.
- [BD97] After the tetrezoitum compound and sodium axide are added to the hemolyzed sample elimpty as they are or as the above-described solution, the printectiment of the hemolyzed sample to carried out, usually by incubating the carried at 40°C to 60°C for 610 72 hours. By pretrecting the sample with the totax clumbrocompound and sodium axide, the measurement sensitivity can be improved as described above, and at the same time, the influence of reducing substances and the like contained in the sample on a redox reaction can be eliminated, thereby improving the accuracy of measurement. Although the totax column compound continuous to the improvement in the accuracy of measurement. Although the totax column axide is present with the bitrax form compound in order to achieve the improvement in measurement sensibility as one of the objects of the present invanition. By using the tetrax column
- compound and sodium saids in combination, an effect peculiar to the present invention can be obtained.

 [0098] Next, the pretreated homolyzed semple containing the tetrazolium compound and sodium saids is treated with a proteise. This proteise treatment is carried out so that a FAOD to be used wherean ect of the ensyle more sailly. Moreover, since the proteise treatment is carried out in the presence of the tetrazolium compound as described above, glycated Ho can be degreeded quickly.
- [0099] As the protonce, serine protessee, theil protessee, metalloproteinasce, and the like can be used, for example. Specifically, trypain, proteiness K, chymotrypain, papein, brometain, subtilish, electrosy, eminopeptidase, and the like can be used. Among these, protessee that degreed the glycated hemoglobin selectively, such as brometain, pepein, trypain derived from percine pencreas, metalloproteinesse, and protesse derived from Backlus subtilish include a product named Protesse N (e.g., Fluis Chemic AG), a product named Protesse N *AMANO* (Amano Enzyme Inc.), and the like, Examples of the metalloproteinesse include
- a product named Protesse N "AMANO" (Amano Enzymo Inc.), and the like, Exemples of the metalloproteinases include metalloproteinases (E.C. 8. 4. 24. 4) derived from the genus Bactiles (e.g., a product named Toyoteam, manufactured by Toyobo Co., Ltd.) and the Ste. Among these, metalloproteinases, bromelain, and papain are more preferable, and metalloproteinases are particularly preferable. Thus, a degradation product of givented his can be prepared selectively

by using a protease that degradus the glycated Hb selectively. The protease treatment usually is carried out in a buller solution, and the conditions of the treatment are determined as appropriate depending on the type of the protease used, the concentration of the checked Hb, etc.

used, the concentration of the glycated Hb, etc.

[0100] As the buffer solution, CHES, CAPSO, CAPS, phosphate, Trie, EPPS, HEPES buffer solutions, and the like can be used, for example. The pH of the buffer solution is, for example, in the range from 6 to 13, preferably from 8 to 12, and range professibly from 9 to 11. Moreover, the final concentration of the buffer solution in the solution subjected to the protected returned is, for example, to the range from 1,0 to 10 mmoV.

[0101] Specifically, when the pretrected hemolyzed sample is treated using a metaloproceinage as the protesse, the protesse treatment usually is carried out under the conditions as follows: the concentration of the metalloproteinage in the machine solution in the range from 0.1 to 40 km/r; the concentration of blood coils in the resection solution in the range from 0.55 to 15 volvs; the receipt in the trange from 1.6°C to 37°C; the reaction period in the range from 1 minute to 24 hours; and the pH in the range from 6 to 12.

(0102) Furthermore, when the protected hemolyzed sample is freeted using proteinade K as the protesse. The protesse treatment usually is carried out under the conditions as follows: the concentration of the protesse in the reaction eclution in the range from 10 to 300 KU/f; the concentration of blood cells in the reaction solution in the range from 0.05 to 15 vol16; the reaction temperature in the range from 15°C to 37°C; the reaction period in the range from 1 minute to 24 hours; and the pM in the range from 6 to 12. Moreover, the type of the buffer solution is not particularly limited, and for example. The-HCL EPPS, PIPES buffer solutions, and the fixe can be used.

[0103] Naxt, the hemolyzed earnple treated with the protesso is treated with a FAOD-a (degradation FAOD) catalyzing the reaction represented by Formula (1) above, more specifically the reaction represented by Formula (7) below.

[0104] In Formula (7), R1 depotes a sugar residue as in the above, and R2 denotes an amino-acid elde chain group

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[0105] By this meatment, the glyceted emino acid having a glyceted e-nmino group and the glyceted e-amino proup of the glyceted Hb degradation product contained in the hemiolyzed sample are degraded.

[0106] According to the FACD-α treatment, among various glyceted amino acids, the one having a glyceted side-

[UTUS] According to this FAOD-a trestment, among various glycated emino acids, the one having a glycated sidechain emino group remains without being degraded. However, considering the ratio of the gyested emino acid having a glycated side-chain amino group to the glycated emino acids as a whole and the ratio of the same to amino acid making the having a glycated side-chain emino group in glycated Hb. It can be said that the influence of the remaining the minimum could be asset or that the course of the same to t

glyceted smirro acid is arrait so that the accuracy of the measurement can be improved sufficiently.

[0107] The FAOD-a treatment is carried out, for example, under the conditions as follows: the concentration of the FAOD-a in the reaction collition in the reaction collition in the reaction collition in the reaction collition in the reaction between the reaction temporature in the renge from 0.5 to 50° C. the reaction temporature in the range from 0.5 to 50° C, the reaction period in the range from 1 minute to 1 hour, and the pH in the range from 6 to 9. The FAOD-a treatment usually is carried out in a buffer solution, and the came buffer solutions as in the processe treatment also can be used in the FAOD-a treatment.

(0108) Subsequently, the hemolyzed completioned with the FAOD-ox is treated further with a FAOD-ox. As described above, the FAOD-ox sets on both a glycated e-emins group and a glycated ide-chain amino group. However, since the glycated Hb degradation product has been treated with the degradation FAOD-ox in advance, it is possible to cause this measurement FAOD-ox to act only on the glycated elde-chain amino group of the glycated Hb degradation product. [0109] Similarly to the above-described protease treatment, this FAOD-ox treatment preferably is carried out in a buffer solution. The type of the buffer solution is not particularly limited, and the same buffer solutions as in the protease treatment.

[0110] The FAOD-oS treatment is carried out, for example, under the conditions as follows: the concentration of the PAOD-oS in the reaction existen in the range from 10 to 30,000 L/I, the concentration of the blood calls in the reaction abullion in the range from 0.10 5 volfs, the reaction period in the range from 20°C to 50°C, the reaction period in the range from 1 minute to 1 hour, and the phi in the range from 6 to 9.

[0111] Next, the hydrogen peroxide formed by the FAOD-as treatment is measured by causing a further redox reaction using an exidese and a color-developing substrate.

[0112] As the color-developing superieto, DA-64, orthophomylaned(emine (OPD), a substrate in which a Trindor's reagant and 4-eminoantipyrine are combined, and the filtercan be used, for example. Examples of the Trindor's reagent include phenots, phonol derivatives, antiline derivatives, naphthols, naphthols, naphthols, naphthols, naphthols, naphthols.

amine derivatives. Furthermore, in place of the aminoantipyrine, it is possible to use aminoantipyrine derivatives, vanillin dismine sufferic scid, methylberzothiazotinene hydrazone (MBTH), autionated methylberzothiazotinene hydrazone (SMBTH), and the like. Among these color-developing substrates, DA-64 is particularly preferable.

[D113] As the oxidazo, a POD preferably is used, for example.

- [0114] The redox reaction mustly is carried out in a buffer solution. The conditions of the reaction are determined as appropriate depending on the concentration of the hydrogen perexide formed, etc. The conditions are usually as follows: the concentration of the POD in the residen solution in the range from 10 to 100,000 IUA; the concentration of the color-developing substrate in the reaction solution in the range from 0,005 to 30 mmot/r, the reaction temperature in the range from 0,005 to 30 mmot/r, the reaction period in the range from 0,1 to 30 minutes; and the pM in the range from 0 to 9.0 Morrower, the type of the buffer solution is not periodiarly limited, and for example, the same buffer solutions as in the protesse treatment and the FAOD treatments can be used.
- [0115] In the redox reaction, for example, when the color-developing substrate is used, the amount of the hydrogen perceited can be determined by macauring the degree of the color developed (i.e. absorbance) in the reaction solution with a spectrophotometer. Then, the amount of the givested his in the sample can be determined using the amount of the hydrogen perceited thus measured and a previously propared calibration curve showing the correlation between an amount of hydrogen perceide and an empount of givested His, for example.
- In amount of hydrogen peroxics are and a removed, the catalytic of example.

 [0116] The hydrogen peroxics and a removed, Thus, it does not have any influence on the measurement in the blood sample (herinolyted example) and is removed. Thus, it does not have any influence on the measurement of the hydrogen peroxide derived from the analyte formed by the FAOD-as. The hydrogen peroxide formed by the FAOD-as are not prevent the hydrogen peroxide formed by the FAOD-as treatment to be performed later from also being removed. It is professible to add excess smoother of POD and color-developing substrate when adding the FAOD-as. In this case, the POD preferably is added so that its activity (U) becomes 3 to 100 times that of the critisace added, in this case, the POD preferably is added as that its activity (U) becomes 3 to 100 times that of the critisace added, in
- [0117] During the degratation FAOD treatment of the homolyzed sample, urlease, bilinchin existsee, and the like, for example, further may be edded to treat the hemolyzed sample in the same manner. By treating the sample with the urlease, it is possible to degrade urle acid contained in the sample. On the other hand, by treating the sample with the billrubin existsee, it is possible to degrade billrubin contained in the sample. The urle acid and billrubin have reducing power. Thus, by treating the sample in the above-described manner, the influence of reducing substances further can be diministed, which allows the accuracy of measurement to be improved.
- [0118] The amount of the unlesse or bilinubin addises ended to the sample is, for example, as follows: when the concentration of blood cells in the reaction solution is in the range from 0.2 to 2 voPk, the universe is added so that its concentration in the reaction solution falls in the range from 0.1 to 5000 UM, partiers that the concentration in the reaction solution falls in the range from 0.5 to 1000 UM, partiers that the concentration in the reaction solution falls in the range from 0.1 to 5000 UM, partiers by from 0.5 to 1000 UM, and more preferably from 2 to 1000 UM, and the conditions of the unlesse of bilinubin axidesse treatment may be the same as these of the degradation 2 to 1000 UM, partiers that the universe of bilinubin axidesse treatment to not necessarily performed before the degradation FACO-a treatment is described above, and may be performed after the FACD-a treatment, for example, As described above, by professe treatment is carried out as that the FACD-a cen act more easily. However, those the FACO-a treatment is carried out in order to degrade the glycated amino actd, the effect of the present invention can be beliated sufficiently
- even if the glycsted Hb is not degraded with the protease prior to the FADD-a treatment.

 [0120] In the method for measurement economic to the present invention, the order of perfamiling the respective treatment shape is not limited to the order described in the present embodiment, and for example, some of the treatment stops may be performed simultaneously. For example, the homolysis treatment, the tetrazoitum compound and acclumnated at the treatment, and the degradation FAOD treatment may be performed simultaneously, or alternatively, the protease breatment and the tetrazoitum compound and acclumnative resident may be performed simultaneously. However, the breatment and the tetrazoitum compound and acclumnative or not timited to these combinations.
 - [U121] The amount of the hydrogen peroxide can be determined not only by the above-described ensymatic method using the POD etc. but also by an electrical method, for example.

(Embodiment A-2)

- [0122] The present embodiment is an exemple where the same FAOD is used to degrade a glyceted emine acid and to measure glyceted Hb. The FAOD used is not particularly limited, and for example, any of a FAOD-a, a FAOD-s, and a FAOD-co may be used. In the present embodiment, measurement is carried out in the same manner as in Embodiment A-1, unless otherwise stated.
- [0123] A hamolyzed sample is pretrested by adding a totmzellum compound and sodium azide thereto, and a dogradation FAOD is added to this pretrested hemolyzed sample.

[0124] Specifically, the conditions of the degradation FAOD inserment are, for example, as follows: the concentration of the degradation FAOD in the reaction actual in the range from 10 to 5000 U/I, the concentration of the blood cells in the reaction exciton in the range from 2.5 to 20 vd%, the reaction temperature in the range from 2.5 to 20 vd%, the reaction temperature in the range from 2.5 to 20 vd%, the reaction period in the range from 2.5 to 20 vd%, the reaction period in the range from 1 minute to 1 hour, and the same buffer abutions as described above also can be used in the treatment. [0125] Next, the sample treated with the degradation FAOD is treated with a protesse. A first object of this protesse treatment is to degrade the glycated Ho derived from blood cells so that a mechanism FAOD to be added tator can set thereon more easity, as described above. A second object of the protesses treatment to tell next value the degradation FAOD to degradation.

[0126] Since FAODs have properties that they act on glycated amino acids more easily than on glycated proteins, the glycated emitto ecid is degraded by the degradation FAOD treatment. However, if the glycated his is treated with the protease in the otics where the degradation FAOD still remains, there effice a protein in that the remaining FAOD reacts with the glycated his cannot be measured accurately. Thus, in order to prevent the remaining FAOD from reacting with the glycated his degradation product, the remaining FAOD is lactivated with the protease. To this and, the amount of the protease to be additionable to be additionable to the content of the protease to be additionable to the degradation FAOD acided first to be inectivated repicity and also the glycated his to be degraded. [0127] The type of the protease is not particularly limited, and the same protease as accombed above also can be used. The conditions of the protease treatment are determined as appropriate depending on the type of the protease used, the concentration of the glycated Hb, the type and the amount of the degradation FAOD, etc.

[0128] The protesse is added so that its concentration in the reaction solution of the protesse treatment falls, for sxample, in the range from 1 to 1,000,000 KU/I, preferably from 10 to 300,000 KU/I, and more preferably from 100 to 100,000 KU/I, when the concentration of the degradation FAOD is 100 U/I.

[0129] Specifically, whon the cample is treated using typesin as the protease, the protease treatment is carried out, for example, under the conditions as histories; the concentration of the protease is the reaction solution in the range from 0.2 to 5 volts; the concentration of the protein in the range from 0.2 to 5 volts; the concentration of the FACD in the reaction solution in the range from 10 to 1000 U/I; the reaction temperature in the range from 20°C to 50°C; the reaction period in the range from 10 minutes to 20 hours; and the pH in the range from 8 to 9.

[0130] Subsequently, the same FAOD as the degradation FAOD is added again as a monsurement FAOD to treat the glycated Hb degradation product obtained by the protesse treatment. It is necessary to edd a sufficient amount of the measurement FAOD because there is a possibility that the measurement FAOD may be inactivated with the protease.

[0131] The measurement FAOD beatment also preferably is carried out in a buffer solution as in the above. The type of the buffer solution is not particularly limited, and the same buffer solutions as in the professe treatment also can be used in this measurement FAOD treatment.

[0132] The measurement FAOD is added as that the concentration in the reaction solution of this measurement FAOD treatment is, for example, in the range from 10 to 1,000,000 U/I, preferably 100 to 200,000 U/I, and more preferably 500 to 50,000 U/I, when the concentration of the protease is 10,000 KU/I.

(0133) Specifically, the conditions of the measurement FAOD treatment are, for example, as follows: the concentration of the necesurement FAOD in the reaction solution in the range from 500 to 20,000 U/I; the concentration of the protease in the reaction solution in the range from 100 to 30,000 KU/I; the concentration of blood cafe in the reaction solution in the range from 0,01 to 1 vol's; the reaction temperature in the range from 1 of the 1 vol's; the reaction temperature in the range from 1 minute to 1 hour; and the pH in the range from 8 to 9.

45 (Embodiment A-3)

[0134] The present embodiment is an example where the same FAOD is used to degrade a glycated amine acid and to measure glycated Mb, in the present embodiment, measurement is carried out in the same manner as in Embodiment A-1, unless otherwise stated.

50 [0135] The present embediment differs from Embodiment A-2 in that it is not always necessary to inactivate a depresent of the control of the process of the present of the process o

It is important to adjust the ristle of a degradation FAOD to a measurement FADD added to a sample as described later.
[0138] A hamolyzed sample is pretreeted by adding a tetrazoitum compound and addium azide thereto, and a degradation FAOD is added to this pretreeted hamolyzed sample.

[0137] When it is difficult to inactivate the degradation FADD with the prohoses used, the degradation FADD needs

to be added in an amount such that, even if the activity of the degradation FAOD remains during the protesse treatment, it does not set on the glycated his degradation product formed. Furthermore, in order to utilize the proporties of FAODs that they set on glycated smino acids easily whoreas they do not set on glycated proteins easily, the amount of the degradation FAOD to be added and the reaction period proferably are set so as to allow the degradation FAOD to act only on the glycated amino acid.

(9138) The conditions of the FAOD freament are, for example, as follows: the concentration of the FAOD in the reaction solution in the range from 10 to 5000 Wi; the concentration of blood cells in the reaction solution in the range from 0.2 to 20 vol %; the reaction temperature in the range from 20°C to 50°C; the reaction period in the range from 1 minute to 1 hour; and the pH in the range from 5 to 9. This treatment usually to carried out in a buffer solution, and the asmo buffer solutions as described above also can be used in this treatment.

[0139] Next, the sample treated with the FACO is treated with a protesse. Since the present embodiment is an example where the protesse handly seds on the FACO, the amount of the protesse to be added a not particular limited. [0140]. The type of the protesse handly seds on the FACO, the amount of the protesse as described above also can be used. The conditions of the protesse treatment are determined as appropriate depending on the type of the protesse trade, the concentration of the givested Hb, and the substrate specificity of the protesse used with respect to the FACO, since the described above.

[0141] Examples of the combination of a FAOD and a proteose failing within the present embodiment include the combination of a product named FOD (Asshi Chemical Industry Co., Ltd.) and a product named Toyoteam (Toyote Co., Ltd.) and the combination of a FAOD derived from the genus Gibbersits and a product named Proteiness K (McTimenn-La Roche Inc.).

[0142] When the sample is treated using type in so the protease, the protease treatment is carried out, for example, under the conditions as follows: the concentration of the protease in the reaction solution in the range from 100 to 8000 U/I; the concentration of blood cells in the reaction solution in the range from 0.2 to 5 voi?s; the concentration of the FACO in the reaction solution in the range from 0.2 to 5 voi?s; the concentration of the FACO in the reaction solution in the range from 10 to 100 U/I; the reaction period in the range from 20°C to 50°C; the reaction period in the range from 0 minutes to 20 hours; and the pM in the range from 8 to 9.

[0143] Subsequently, the same FAOD as the degradation FAOD is added again as a measurement FAOD so that a sets on the glycated Hb degradation product obtained by the protesse treatment.

[0144] The measurement FACO treatment also preferably is carried out in a buffer solution as in the above. The type of the buffer solution is not particularly limited, and the same buffer solutions as in the proteose treatment also can be used in this measurement FACO treatment.

[0145] Thus, in this present embediment, the ratio (activity ratio A: B) of the degradation FACD (A) to the measurement FACD (B) added to the sample is set, for example, in the range from 1 : 50,000 to 1 : 10, preferably 1 : 500 to 1 : 50, as described above. In the present embediment, the degradation FACD remains in the reaction solution untike Embodiment A2. However, when the ratio is in the above-described range, the remaining degradation FACD does not act on the glycated Hb degradation product during the proteined treatment to such an extant that it affects the measurement because the reaction ratio of the remaining degradation FACD is very low. [1145] The conditions of the measurement FACD instrument are, for example, as follows: the concentration of the measurement FACD in the reaction about on in the range from 100 to 30,000 KU/t; the concentration of thod calls in the reaction solution in the range from 100 to 30,000 KU/t; the concentration of tood calls in the reaction solution in the range from 0.01 to 1 vol%; the reaction temperature in the range from 0.01 to 1 vol%; the reaction period in the range from 1 intrute to 1 hour; and the pH in the range from 8 to 9.

(Embodiment A-4

- 45 [0147] The present embodiment is an example where hydrogen peroxide formed by the measurement FACO treatment described above is measured using DA-84 at a color-developing substrate, in the present embodiment, measurement is carried out in the same menner as in Embodiment A-1, unless otherwise stated.
 [0148] When DA-84 is used as the color-developing substrate, in order to prevent the color development error of the
 - [0148] When DA-64 is used as the color-developing substrate, in order to provent the color development error of the DA-64, final concentrations of the DA-64, the tetrazoitum compound, the accidum axide, and surfaceant in the feet action of the redox reaction are set in the fellowing ranges. Usually, the final concentration of the DA-64 is 1 to 10,000 µmoV; the variactant is 0.01 to 200 mmoV; the tetrazoitum compound is 0.05 to 20 mmoV, and the sodium axide is 0.01 to 5 mmoV, the final concentration of; the DA-64 is 2 to 1000 µmoV; the surfactant is 0.05 to 30 mmoV i; the tetrazoitum compound is 0.01 to 10 mmoV; and the accium axide is 0.02 to 2 mmoV, the final concentration of: the DA-64 is 3 to 300 µmoV; the surfactant is 0.1 to 10 mmoV; the tetrazoitum compound is 0.5 to 8 mmoV; and the accium axide is 0.02 to 2 mmoV. The type of the surfactant is not particularly limited, and these
 - described above and below may be used, for example.

 [0149] This pit of this reaction solution preferably is in the range from 8.0 to 10.0, more preferably from 8.5 to 9.0, and particularly preferably from 7.0 to 8.5.

(0150) The surfactant may be added, for example, either before adding the DA-64 or simultaneously with the DA-64. When the surfactant is added to propere a homolyzed sample, the surfactant may be added in advance so that its concentration fulls within the above-described range when the redox reaction occurs.

(0151) The DA-64 develops color by a radex reaction. Thus, by measuring the absorbance (i.e., the degree of the color developed) of the reaction satisfies with a spectrophotometer, for example, at a wavelength in the range from 600 to 780 nm, the amount of the hydrogen perexide can be determined.

(Embodiment A-6)

of two or more types.

- (0152) Herehafter, the method of determining a ratio of glycated His to total his according the present invention will be described in defail with reference to the following example, in which whole blood is used as a sample.
 - [0153] First, whole blood is hemolyzed to prepare a hemolyzed sample and the amount of glycated Hb in the sample is measured in the same manner as in Embediment A-1. On the other hand, the amount of Hb in the sample also is measured.
- [0184] The amount of Hb can be measured in the following manner, for exemple. First, the tebracellum compound as described above is added to the hemotysate sample to densture the Hb. For exemple, when the concentration of blood cells in the hemotysade sample is in the range from 0.2 to 2 volks, it is preferable that the totrazolium compound is added so that the concentration fells in the range from 0.005 to 400 mmoW, more preferably from 0.02 to 100 mmoW is added so that its concentration fells in the range from 0.004 to 16 mmoW, more preferably from 0.1 to 50 mmoW. Specifically, when the latrazolium compound is WST-3, preferably it is added so that its concentration fells in the range from 0.004 to 16 mmoW, more preferably from 0.02 to 10 mmoW.
 - [D188] Affhough the tetrazollum compound may be used simply as it is, it preferably is used as a solution in which the tetrazollum compound is dissolved in a solvent, in terms of simplicity in operation, officionary of the tetrazollum compound (a.g. molecular weight or the solution can be determined as repreparities depending on the type of the tetrazollum compound (a.g. molecular weight or the Sko), otc. For example, the concentration is in the range of 0.01 to 120 mmol/l, preferably from 0.1 to 50 mmol/l, and more preferably from 0.2 to 20 mmol/l. As the solvent, for example, the series buffer solutions are mentioned above can be used. Moreover, the tetrazollum compound may be used abline as mentioned above can be used. Moreover, the tetrazolium compound may be used abline along or in combinations
- so [U186] The conditions of the treatment with the tetrazollum compound are not perticularly limited, but may be as follows, for example: the temperature in the range from 4°C to 50°C and the treatment period in the range from 20 seconds to 60 minutes; preferably, the temperature in the range from 15°C to 40°C and the treatment period in the range from 20 seconds to 20 minutes; and more preferably, the temperature in the range from 25°C to 37°C and the treatment period in the range from 30 seconds to 8 minutes.
- 15 (0157) This (ctrazellum compound treatment preferably is contact out in the presence of a surfactant at described above, because this can accelerate the denaturation of the Ho still further. To this end, the tetrazellum compound may be added to the sample during the hemolysis treatment for simplicity in operation and the like.
- [ot 58] As the surfactant, polytoxyethylene sthem, polytoxyethylene phenol eithers, polytoxyethylene activities alkyl estate, polytoxyethylene alkyl estates, polytoxyethylene alkyl estates, polytoxyethylene alkyl estates, and the like can be used. Among these, polytoxyethylene alkyl estates, which are represented by [CtH_xO (CH_xCH_xO)_x/th], are compounded in which a polytoxyethylene ahalin and a hydrocarbon chail are inched with each other by whiter linkage. Examples of the hydrocarbon chail nicities and an alkyl group and an alkyl phenyl group. Profombly, the weight-average degree of polymerstation (N) of the polytoxyethylene chain is in the range from 8 to 23 and the carbon number (L) of the hydrocarbon chain is in the range from 8 to 18; more proforably, the weight-average degree of polymerstation (N) is in the range from 8 to 15 and the carbon number (L) is in the range from 8 to 16; more proforably, the weight-average degree of polymerstation (N) is in the range from 8 to 10 and the carbon number (L) is in the range from 8 to 14. For example, the hydrocarbon chain may be a alreight chain or may have a brenched chain. Specific examples of the polytoxyethylene-pt-cotyphenyl ether, polytohylane glycol [10] suryl ether, and polytoxyethylane-pt-cotyphenyl ether, polytohylane glycol [10] suryl ether, and polytoxyethylane glycol [10]
- TOTS9] More specifically, polycoyethylene-p-t-octylphanyl other such as commercially available. Thisn series surfactants; polycoyethylene sorbitan alkyl seter such as commercially evailable. Twoch series surfactants and polycoyethylene alkyl other such as commercially evailable. Twoch series surfactants can be used. Other than these, polycoyethylene (9) launyl ether; polycoyethylene (9) launyl ether such as a product named Nikkel BL-9 EX (the weightseverage degree of polymertestion (N) of polycoyethylene is 9, manufactured by Walke Pure Chemical Industries, Ltd.); and polycoyethylene octylphenyl ether such as a product named Torgitot NPX (the weight-sverage degree of polymertestion).
 - new polytocytem in the control of th

[0160] The amount of the surfactum added to the hemotyzed sample is not perfocularly limited. However, when the concentration of blood cells in the sample to in the range from 0.2 to 1 vol%, it is preferable that the surfactant is added so that be concentration falls in the range from 0.06 to 50 mmol/l, more preferably from 0.2 to 30 mmol/l, and particularly preferably from 0.3 to 10 mmoV. Specifically, when the surfactant is a product named Triton X-100, preferably it is added so that its concentration falls in the range from 0.2 to 100 mmoV, more preferably from 1 to 30 mmoV, and particularly preferably from 2 to 20 mmol/l. When the surfactant is a product named Bril 85, preferably it is added so that its concentration fells in the range from 0.1 to 50 mmoVI, more professibly from 0.5 to 20 mmoVI, and particularly

The surfactant may be added to the sample so that, when 0.5 to 5 mmel of the tetrazolium compound is preferably from 1 to 10 mmol/l. present, 0.1 to 70 mmol, preferably 0.3 to 50 mmol, and particularly preferably 0.4 to 20 mmol of the surfactant to present, for example. Specifically, when the surfactant is a product named Thion X-100, it preferably is added to the sample so that, with respect to 1 mmol of the tetrezollum compound, 0.2 to 15 mmol, more preferably 0.5 to 10 mmol, sample so that, with respect to 1 miles of the terrecipient compound, u.e. to 10 miles, may proceed up to 10 miles, and particularly proferably from 0.7 to 5 minol of the surfectant is present. When the surfectant is a product named Brit 35, it preferably is added to the semple so that, with respect to 1 minol of the tetracolum compound, 0.1 to 10 minol, one preferably 0.2 to 8 minol, and particularly preferably more preferably 0.2 to 8 minol, and particularly preferably more 0.3 to 4 minol of the surfactant is present, it is to be more presently $v \ge n$ is minor, and particularly proteined from v = v is amount of the surfactant for accelerating the noted that, during the hamolysis treatment of the sample, a sufficient amount of the surfactant for accelerating the denaturation of the Hb may be added in selvance.

[D182] Next, the absorbance of the denatured Hb is measured. It preferably is measured at a wavelength in the range

Iram S20 to 970 mm, more preferably from 650 to 660 nm, as documented above. When the dual-wavelength measurement intimezono or a minimize previously managed to occurring a copia serior and unconservative and interest measurement. Is carried out using the wavelength in the above-mentioned range as the main wavelength, the sub-wavelength preforably is in the range from 730 to 900 nm, more proferably from 800 to 900 nm, and particularly preferably from 900

[0163] Using the thus measured absorbance of the denatured Hb and a calibration curve prepared in advance, the

[0162] Using the thus-measured absorbance of the described Hb and a calibration curve prepared in advance, the amount of the Hb its determined. Then, using the amount of the glycated Hb measured in the above and the amount of the Hb thus determined, the ratio of the glycated Hb to the Hb can be calculated. First, standard solutions containing different known emounts of Hb are provided. The emounts of Hb in these standard solutions are measured by the ling different known emounts of Hb are provided. The emounts of Hb in these standard solutions are measured by the above-described method of measuring Hb. Then, based on the obtained above-described method of measuring Hb. Then, based on the obtained measured values, the calibration curve is prepared. The known method is not specifically limited as long as it can measured values, the calibration curve is prepared. The known method is not specifically limited as long as it can measure Hb with high accuracy. However, the HiCN method as an intermidence standard method is preferable, for examples.

[0185] To carry out the absorbance measurement of the denatured Hb to determine the amount of the Hb, part of the hemotyzed sample for messauring the giyosted Hb may be baken to preper a semple for messauring the Hb. How the memory consequence of the absorbance measurement as part of the processes for measuring the phycated Hb. because the measurement can be carried out quickly and simply. Specifically, in the measurement of the phycated He became the measurement and or example, it is possible to measure the absorbation of the densitured Hb siter the addition of the tebezolium compound to the hemotyzed sample, after the addition of the tetrazolium compound and the section exide to the hemolyzed sample, after the protease treatment, after the measurement FADD treatment, or

(Embodiment A-6)

[0185] Hereinalier, the method of measuring HbA1c according the present invention will be described in detail with

(0166) Hereinafter, the method of measuring HbAta according the present invention will be described in detail with reference to the following example, in which whole blood is used as a sample.

[0167] First, a whole blood sample is provided, and the amount of glycetoid Hb in the earnple to measured in the serior mannor as in Embodiment A-1. On the other hand, various glycetoid Hb in standard solutions, in such of which an earner of HbAta in glycetoid Hb in stood of these standard solutions amount of HbAta in glycetoid Hb in thrown, are provided. The amount of glycetoid Hb in stood of these standard solutions is measured in the above-described manner. Then, a calibration curve is prepared that shows the relationship between the measured valve (the amount of phychod Hb) and the amount of HbA1c in these standard solutions. As described shove, there is a correlation between the measured value of the glycated Hb and the amount of HbA1c. Therefore, by soons, mere is a consistent conviction and measured value of the greatest to allow this calibration curve, the amount substituting the measured value of the givested His in the whole blood sample line this calibration curve, the amount

of HbA1c in the whole blood sample can be determined. (0158) In the proparation of the collection curve, the measured value of the amount of glycated his is not limited to to value abtained finally, and may be an absorbance of the reaction colution of the POD treatment in the process for

measuring the emount of glycated Hb, or an amount of hydrogen perceids determined using this absorbance.

[169] Next, a measuring kit according to the present invention will be described in detail with reference to the following example, in which the analyte is glycsted Ha.

(Embodiment B-1)

[0170] The present ombodiment is one example of the first measuring kit, in which a FAOD-or is used as a degradation contained in a pretreatment reagent and a FACD-o3 is used as a measurement FACD conteined in a color

[0171] This measuring kill includes a pretreament reagent containing a degradation FAOD, a protrease reagent con-(thing a protegeo, and a color-developing reagent containing a measurement FACO, an oxidereductase, and a color-

[0173] Each of the protreatment reagent, the protests reagent, and the color-developing reagent can be prepared by dissolving the component(s) to be contained therein in an equeous solvent.

(Protrostment Reagont)

[0173] In the color-developing reaction equation obtained finally, the pretreament resignit is diluted 10-fold to 200-fold, pretarebly 20-fold to 100-fold, for example. Therefore, the concentration of the FAOD and the like contained in this pretreatment reagent can be determined as appropriate depending on this diluting factor etc., for example, in this pretreatment reagent can be determined as appropriate depending on this diluting factor etc., for example, furthermore, the emount of the FAOD and the like in the reaction system may be adjusted by changing the smouth of the FAOD and the like in the reaction system may be adjusted by changing the smouth of the faot of the factor o the pretroitment reagent to be added to a sample depending on the amount of an analyte or a givested amino add to be degraded contained in the eample.

Specifically, the concentration of the degradation FAOO in the pretreatment reagant is in the range from 10 [0174]

[0175] As the equocus solvent, afflough not perticularly limited, water, buffer solutions containing the above-de scribed various buffers, and the like can be used, for example, Among the above-described buffers, CHES, MOPS. window vinkture presents, and the tike over the country, for example, which is accountreashing present of the propriete TAPS, EPPS, phosphiete, HEPPSO, POPSO, and bomte are preferable, and CHES, MOPS, TAPS, and phosphiete ero more preferable. The concentration of the buffer solution is, for exemple, in the range from 5 to 200 mot/l, preferably 20 to 160 molf. Furthermore, the pH of the buffer solution is, for exemple, in the range from 8 to 10, preferably 8.5 to 10. [0177] This precentment reagon further may contain the following components in addition to the degradation FAOD, [0177] When the pretreatment reagent contains a surfactant, various surfactants as above described can be used, for example. However, errong these surfactants, polyoxyethylene skyl others and polyoxyethylene octyphenyl others are preferable. The concentration of the surfactant is, for example, in the range from 0.63 to 200 minor, preferably

[0178] When the pretree/ment roagent contains uricese, is concentration to in the range from 1 to 2000 UA, for example. On the other hand, when the pretreatment reagent contains billitudin oxidase, to concentration is in the range from 1 to 2000 LM, for example. The protreatment reagent may contain both uncase and billitudin oxidase.

[0179] In the color-daveloping solution obtained finally, the protesto reagont is cliuted 1.1-fold to 3-fold, for example. Therefore, as in the case of the pretreatment reagent described above, the concentration of the respective components in this protease reagent can be determined as appropriate depending on this cituding factor etc. Furthermore, the amount of the professe in the reaction system may be adjusted by changing the amount of the professe reagent to be added to a sample depending on the amount of glycated Hb in the sample of.

[0180] As the protesses, the above-described protesses can be used, for example, When the protesses is a metalloturisus. As the processes, the concentration of the processes the processes the concentration of the processes in the processes congest is, for example, in the earge from 0.5 to 100 MM/

[0181] As the equeous servers, attrough not persoularly limited, water, buffer sofutions containing the above-deecribed various buffers, and the like can be used, for externels, Among the above-described buffers, MES and MOPS earlies are preferable, and MES is more preferable. The concentration of the buffer satisfacts is, for example, in the range from 1 to 20 mmol/l, preferably 1 to 5 mmol/l. The pH of the buffer solution is, for example, in the range from 5.0 to 7.0. prefembly 5.5 to 8.6.

[0182] This protesse reagent further may contain the following components in addition to the protesse.

[0183] Examples of the components other than the protesse include a totazzellum compound and sodium azide. When the protoses respent further contains a tetrezollum compound and sodium azido, the concentrations of these components are, for example, as follows: the concentration of the tairszollum compound is in the range from 0.1 to 10 movi and the concentration of the sodium saids is in the range from 0.05 to 4 mmovi; preferably, the concentration of the sodium saids is in the range from 0.6 to 5 mmovi and the concentration of sodium saids is in the range from 0.6 to 5 mmovi and the concentration of sodium saids is in the range from 0.15 to 1.8 mol/l. Furthermore, the tatrazolium compound (C) and the codium azide (D) preferably are added so that they are present at a ratio (moter ratio C:0) in the range from 20:3 to 20:12, more preferably from 20:5 to 20:

11, and particularly preferably from 20 : 6 to 20 : 10.

[0184] When the protecce is a metalloproteinase, the protects reagant further may contain a Ca compaund and a VITES I Winen the protected is a metalloproteinase, the protease reagant further may contain a Ca compound and is Na compound. The Ca compound and the Na compound are not particularly limited as long as they knize to Ce² and Na*, respectively, in the equeeus selvent. As the Ca compound, calcum chiedde (CeCl₂), CeSO₂, (CH₂COO)₂Ca, and the like can be used, for example, Among these, CaCl₂ and CeSO₄, are protectable. On the other hand, as the Na compound, sodium chiedde (NaCl), CH₂COON₂Ca, NaNO₃, NaNO₃, and the like can be used, for example, Among those, NaCl₃ and CaSO₄, NaNO₃, and the like can be used, for example. these, NaCl and Na₂SO₄ are preferable. The Ca compound and Na compound may be used either atone or in combi-

nations of two or more types.

[0185] The concentration of the metaloproblemsels, for example, in the range from 0.5 to 100 MUA, preferably from 1 to 40 MUA, as described above.

[0186] The concentration of the Ca compound is in the range from 0.1 to 50 mmol/s to that the concentration of dissociated Ca²⁺ becomes 0.1 to 5 mmol/s. On the other hand, the concentration of the Na compound is in the range from 5 to 1000 mmol/s, preferably from 10 to 300 mmol/s, so that the concentration of dissociated Na²⁺ becames 5 to 1000 mmol/s, preferably from 10 to 300 mmol/s, so that the concentration of dissociated Na²⁺ becames 5 to 1000 mmol/s. 1000 mmol/s.

(Color-developing Reagent)

[0187] In the color-developing solution colorined finally, the color-developing reagent is diluted 2-fold to 20-fold, preferably 3-fold to 10-fold, and more proferably 4-told to 8-fold, for example. Therefore, as in the case of the abovedescribed reagants, the contraction of the respective components in this color-developing reagent can be determined as appropriate depending on this clinting factor etc., for example. Furthermore, the amount of the respective componants in the reaction system may be adjusted by changing the amount of the color-developing reagent to be edded to a sample depending on the amount of glycated His in the sample or the emount of the hydrogen peroxide formed. The concentration of the measurement FACD in the color-developing reagent is, for example, in the range

[0183] The concentration of the extereduction in the color-developing reagent is, for example, in the range from 1 to 1000 KU/I, preferably from 10 to 200 KU/I, As the addoreductase, a POD can be used, as described above, to 1000 KU/I, preferably from 10 to 200 KU/I, As the addoreductase, a POD can be used, as described above.

[0190] The concentration of the color-developing substrate in the color-developing reagent is, for example, in the range from 0.001 to 100 mmoVI, preferably from 0.005 to 10 mmoVI, and more preferably from 0.02 to 1 mmoVI. As

the color-developing substrate, the above-described substrates can be used, [0191] As the aqueous selvent, ethnough not particularly limited, water, buffer solutions containing the above scribed various buffers, and the like can be used, for example, Among the above-described buffers, The and phosphate

are professile. The concentration of the buffer solution is, for example, in the range from 20 to 1000 mmol/l, preferably 50 to 500 mmol/l. The pH of the buffer solution is, for example, in the range from 8 to 9, preferably 8.5 to 8.

(Mathod of Using Measuring Kit)

[0192] Heroknafter, a method of measuring discated Hb in blood cette using such a measuring kit will be described

[0193] A hemolyzed earnple is prepared from whole black in the same manner as in Embodiment A-1, and the pretreatment reagent is added to this homolyzed sample so that the degradation FAOD soits on a glycated amino acid

in the earlies in regimes a.

[0194] By the action of this degradation FAQD-rdS, the glycalod armine solid having a glycated e-armine group and the glycated destrains group of the glycated Hb degradation product contained in the harnolyzed comple are degraded. According to the FAOD-a treatment, among various glycated entire saids, the one having a glycated side-chain amino group remains without being degraded. However, considering the ratio of the glycated emine acid having a glycated group remains warrout our if degraded, introduced, to replace in a fine table of the same to and pecking decking having side-child amino group to the ghypated entino acid est and so a whole and the ratio of the same to and no acid residues having

side-chain amino group to the objected emino acids as a whole and the ratio of the same to antico acid residues having a glycated eldo-chain amino group in glycated hib, it can be exid that the influence of the remaining glycated amino acid is amall so that the accuracy of the measurement can be interpreted sufficiently.

[0185] The amount of the protreatment reagont to be added is, for example, as follows: to 30 µl of a hemotyzed sample containing 30 to 80 volls of blood cells, 300 to 3000 µl, preferably 800 to 2400 µl of the pretreatment reagont is added, in the protreatment reaction solution obtained by adding the protreatment object, it is preferable that 10 to 5000 Umoli of the degradation FAOD is present, with respect to 1 volls of blood cells. Furthermore, the prior this reaction solution preferably is in the mange from 8 to 10, more preferably from 8.5 to 10.

[0196] In this pretreatment, it is preferable that the sample is incubated after the pretreatment reagant is added thersto. The sample may be incubated, for example, et 10°C to 3°C for 0.1 to 20 minutes, preferably et 16°C to 3°C

[0197] When the pretrestment respect contains a surfactant, it is not necessary to propers a hemolyzed sample by

performing the above-described harmolysis trestment separately, for example. That is, by adding the preince s a whole blood sample or a blood cell sample, both the homotype treatment by the surfactant and the degredation treatment of the glycated amino acid by the degradation FAOD can be performed simultaneously.

[0196] In this case, in the pretreatment reaction exhition obtained by adding the protreatment reagent, it is preferable unit 0.05 to 50 mmoV, more protectibly 0.2 to 80 mmoVI, and periodicity preferably 0.3 to 10 meV of the surfactant is

[0193] When the protresprient reagont contains uricess or blürubin caldisce as described above, the pretresprients when the promeagnent reagent contains unlesse or billrubin exidese as described above, the pretreatment reagent pretreatly contains the unlesse or the billrubin exidese or that, in the pretreatment rescribin solution, 0.4 to 4000 U/I, more preferably 3 to 1500 U/I, and particularly preferably 5 to 1000 U/I of the unlesse or 0.4 to 4000 U/I, more preferably 3 to 1500 U/I, and particularly preferably 3 to 700 U/I of the unlessed, with respect to 1 vortices.

[0200] Next, the processe reagant is added to the protrestment reaction solution so that the degradation FAOD ects

on gryceuro not in the earnine to degrado it.

[0201] The amount of the probate reagent to be added is, for example, as follows: to 10 µl of the protesse reagent is added, reaction solution containing 1 vol% of blood coits, 50 to 300 µl, preferably 90 to 180 µl of the protesse reagent is added. In the protesse reaction solution obtained by adding the protesse reagent, it is preferable that 100 to 50,000 KU/l, more protessely 300 to 30,000 KU/l of the protesse is present, with respect to 0.1 vol% of blood cells. Furthermore, the pKI with respect to 0.1 vol% of blood cells. of this processo reaction solution preferably is in the range from 8 to 9, more professibly from 7 to 8.5.

[0202] In this protection treatment, it is preferable that the sample is incubated after the protesse reagont is added LICEMS. In this processes usuamons, it is preferable that the earning is shoulded about the processe resigning about the cample may be incubated, for example, at 25°C to 37°C for 3 to 30 minutes, preferably at 25°C to 37°C. for 3 to 10 minutes, and more preferably at 30°C to 37°C for 3 to 5 minutes.

[0203] When the proteste reagent combines a totracollum compound and audium azide as described above, their concentrations in the protease reaction acturion are, for example, as follows: when the concentration of the protease is in the range from 100 to 10,000 KU/I, the concentration of the totrazolium compound is in the range from 0.1 to 10 nunoi/ and the concentration of the codium exists is in the range from 0.08 to 4.0 mmo//; preferably, the concentration of the tetrezollum compound is in the range from 0.8 to 5 mmol/l and the concentration of the sodium azide is in the range from 0.15 to 1.8 mmol/l. Specifically, when the tetrezollum compound is WST-0, its concentration in the protesse reaction solution preferably is in the range from 0.2 to 6 mmol/s, more preferably from 0.8 to 4 mmol/s, and perdicularly reaction solution preferably 0.7 to 2.7 mmol/s. By edding the tetracolium compound and the sodium action as described above, the segrement sensitivity becomes about 1.2 to 3 times greater than in the case where they are not added.

(1004) When the processe region contains a metallopreteiness and further conteins a Ca compound and a Na compound se described above, their concentrations in the process reaction solution are as follows: when the concentrations in the process reaction solution are as follows: when the concentration of the metalloproteiness is in the range from 100 to 10,000 KUA. for example, the concentration of the Ca compound is in the prage from 0.1 to ED minet/A and the concentration of the Na compound is in the range from 0.1 to ED minet/A and the concentration of the Na compound is in the range from 0.1 to ED minet/A and the concentration of the Na compound is in the range from 0.1 to ED minet/A and the concentration of the Na compound is in the range from 0.1 to ED minet/A and the concentration of the Na compound is in the range from 0.1 to ED minet/A and the concentration of the Na compound is in the range from 0.1 to ED minet/A and the concentration of the Na compound is in the range from 0.1 to ED minet/A and the concentration of the Na compound is in the range from 0.1 to ED minet/A and the concentration of the Na compound is in the range from 0.1 to ED minet/A and the concentration of the Na compound is in the range from 0.1 to ED minet/A and the concentration of the Na compound is in the range from 0.1 to ED minet/A and the concentration of the Na compound is in the range from 0.1 to ED minet/A and the concentration of the Na compound is in the concentration of the Na compound in the Na compound is in the concentration of the Na compound in the Na compound is in the concentration of the Na compound in the Na compound is in the concentration of the Na compound in the Na compound is in the concentration of the Na compound in the Na compound is in the Na compound in the Na co 1000 mmoVI; preferably, the concentration of the Ca compound is in the range from 0.2 to 10 moV and the concentration of the Ca compound to the Na compound is in the range from 10 to 500 moVI; and more preferably, the concentration of the Ca compound of the Na compound is in the range from 10 to 500 moVI; and more preferably, the concentration of the Ca compound

is in the range from 0.2 to 5 may and the concentration of the Na compound is in the range from 30 to 500 mot. (0205) Next, the color-developing reagent is added to the pretreatment reaction solution so that the measurement FAOD acts on the glycated Hb in the semple, thereby equiling the reaction represented by Formula (1) to form hydrogen peroxide. Then, a radox reaction is caused between the color-developing substrate and the hydrogen peroxide, thereby

causing the color-developing substrate to develop color by exidation. (0206) The measurement FACO-oc acts on both a glycated e-emilino group and a glycated side-chain emitro group. mod above. However, since the glycoted a smillio group is degraded in advance with the degradation FAOD-

a contained in the protreement reagent, it is possible to cause the measurement FACO-as to act only on the glycarad [0207] The arrount of the color-developing reagont to be added is, for example, so follows: to 100 µl of the protesse reaction solution, for example, 5 to 100 µL preferably 6 to 60 µL, and more preferably 10 to 30 µL of the contradevolping reaction solution, for example, 5 to 100 µL preferably 6 to 60 µL, and more preferably 10 to 30 µL of the contradevolping reaction solution obtained by adding the color-developing reaction solution obtained by adding the color-developing reagent, it is preferable to the color-developing reaction solution obtained by adding the color-developing reagent, it is preferable.

reagens in expension of the measurement FADO is in the range from 0.5 to 200 KUA, the concentration of the arrived that the concentration of the measurement FADO is in the range from 0.5 to 200 KUA, the concentration of the color-developing autostrate is in the oxidoreductase in in the range from 1 to 1000 KUA, and the concentration of the color-developing autostrate is in the range from 0.001 to 100 mmo/l. It is more preferable that the concentration of the measurement FACO is in the range from 1 to 100 KU/I, the concentration of the oxidoreductase is in the range from 6 to 200 KL/I, and the concentration of the color-developing substrate is in the range from 0,006 to 10 mmol/l. It is stu more preferable that the concentration of the measurement FAOD is in the range from 2 to 100 KU/I, the concernration of the existence uctions is in the range from 5 to 200 KU/I, and the concentration of the color-developing substrate is in the range from 0.01 to 1 march/ Furthermore, the pH of this color-developing reaction abdulon preferably is in the range from 8 to 9, more professibly

[U208] In this color-developing reaction, it is preferable that the sample is incubated for a predetormined period after

the protesse respont is added thereto. The sample may be incubated, for example, at 15°C to 37°C for 1 to 30 minutes,

the protests respond is according to the degree of the color developed (i.e. absorbance) in the color-developing substrate is measured. This can be measured, for example, by measuring the degree of the color developed (i.e. absorbance) in the color-developing resction exhibits with a spectrophotometer. Then, using this absorbance is the hydrogen perceits a determined. The amount of spectrophotometer. Then, using this absorbance, the amount of the hydrogen perceits distributed the state described and a second color of the color-developing the degree of the color-developi specific procedurates, small, using this absorbances, and enrount of the hydrogen paroxide thus determined and it is smouth of the hydrogen paroxide thus determined and it bleationals bloosted celipsegou onto spanning the consistion pelwast on smorth of physiodes becaute and su smorth

or grydated no, for example.

[2210] The hydrogen perexide formed by the degradation FACO-cs added first reacts with catalase present originally [2210]. The hydrogen perexide formed by the ensured. Thus, it does not have any influence on the measurement of in the blood sample (hemolyzed sample) to be removed. Thus, it does not have any influence on the measurement of the blood sample (hemolyzed sample) to be removed. Thus, it does not have any influence on the measurement of the blood sample (hemolyzed sample) and the hydrogen perexide derived from the analyte formed by the FACO-cs. However, in order to remove the hydrogen me mycrogen perexiste derived from the emerge formed by the Processor. Hereard, in which the hydrogen perexide is formered percently, the protrectment resigner may further contain established. When the hydrogen perexide is formered by the reaction with catalase, in order to prevent the hydrogen perceited formed by the treatment with the measurement FACID-QS contained in the color-developing reagent to be exided lear from rises being removed by the catalase, it is

FACE as contained in the color-developing reagent to be added later from riso being removed by the caldese, it is proferable that the color-developing reagent contains excess amounts of POD and color-developing substrate.

[U211] In this case, the concentration of the catalises in the profestment reagent is, for example, is the range from 5 to 300 U/I, proferably from 10 to 100 U/I, and more preferably from 10 to 70 U/I. Furthermore, the concentration of the catalase in the protractment reaction solution is, for example, in the range from 1.5 to 50 U/I, preferably from 1.5 to 30 U/A, and more preferably from 1.5 to 15 U/A, Still turther, the concentration of the catalese in the color-developing reaction solution is, for example, in the range from 1 to 50 U/I, preferably from 1 to 30 U/I, and more preferably from 1

[0212] The concentration of the POD in the color-developing reagent is, for example, in the range from 5 to 1000 (0212) The concentration of the KLM, preferably from 10 to 200 KLM, and more preferably from 20 to 200 KLM. Furthermore, the concentration of the POD in the color-developing reaction solution is, for example, in the range from 3 to 300 KU/I, preferably from 5 to 200 KU/I, and more proferably from 10 to 100 KU/I.

KUII. and more presently from 10 to 100 KUII.

[0210] The critic (activity ratio E : F) of the critalese (E) to the POD (F) in the color-developing reaction solution is, [0210] The critic (activity ratio E : F) of the critalese (E) to the POD (F) in the color-developing reaction solution is, [0210] The concentration of the color-developing substrate in the color-developing reaction, for example, in the

range from 0.01 to 200 mo/l, preferably from 0.02 to 20 mol/l, and more preferably from 0.04 to 5 mol/l. Furthermore, range from 0.01 to 200 mol/l, preferably from 0.02 to 20 mol/l, and more preferably from 0.04 to 5 mol/l. Furthermore, range from 0.01 to 200 mol/l, preferably from 0.02 to 20 mol/l, and more preferably from 0.04 to 5 mol/l. Furthermore, range from 0.01 to 200 mol/l, preferably from 0.02 to 20 mol/l, and more preferably from 0.04 to 5 mol/l. Furthermore, range from 0.01 to 200 mol/l, preferably from 0.02 to 20 mol/l, and more preferably from 0.04 to 5 mol/l. Furthermore, range from 0.01 to 200 mol/l, preferably from 0.02 to 20 mol/l, and more preferably from 0.04 to 5 mol/l. Furthermore, range from 0.01 to 200 mol/l, preferably from 0.02 to 20 mol/l, and more preferably from 0.04 to 5 mol/l. Furthermore, range from 0.01 to 200 mol/l, preferably from 0.02 to 20 mol/l, and more preferably from 0.04 to 5 mol/l. Furthermore, range from 0.04 to 5 mol/l. F

rongs from 0.001 to 100 mmol/l, preferably from 0.005 to 10 mmol/l, and more preferably from 0.01 to 1 mmol/l.

[0215] In the measurement using the measuring kit, the order of adding the respective regards is not finited to the described order, and some of the reagents may be added simultaneously. Specifically, after the pretreatment responses to the process reagent and the color-developing reagent may be added to the earnife simultaneously, specifically in process reagent and the color-developing reagent may be added to the earnife siter the protectment reagent and the protesse reagent or the color-developing reagent and the protesse reagent. or one cover-developing respons may be educe to the earlies also the processing in a sided to the sample, the process, an added simultaneously, for example, Attendibility, offer the process reagent is added to the sample, the process. or account as minimized by the man place of the control of the man was a second of the man and then the color-developing reagent ray be edded, for example.

ment reagent and then the color-developing reagent may be edded, for exemple.

[0216] Moreover, by using a measuring this according to the present invention, the amount of HbAtc can be measured using the quickly and simply, and with high accuracy as well. This is necessed an amount of HbAtc. Specifically, the amount of HbAtc can be deformined by preparing a calibration curve beadd on the correlation between an emount of glycated Hb measuring the measuring kit of the present invention curve beadd on the correlation between an emount of glycated Hb measuring the measuring kit of the present invention and an amount of HbAtc, measuring the amount of HbAtc, measuring the amount of HbAtc, measuring the present invention and an amount of HbAtc, measuring the amount of HbAtc, measuring the present invention, and these submitted in the measured value into glyceted Ho in a sample by the measuring kit of the present invention, and then substituting the measuring in the measuring kit of the present invention, and then substituting the measuring kit of the present invention, and then substituting the measuring kit of the present invention, and then substituting the measuring kit of the present invention, and then substituting the measuring kit of the present invention, and then substituting the measuring kit of the present invention. this officerion curvo.

(Embodiment 8-2)

[0217] The present embodiment is one example of the second measuring kit, in which the same FAOO is used as a Learn I have become appropriately as the exemple of the appeal measurement FAOD contained in a color-developing degradation FAOD contained in a color-developing regradation PAQU contained in a pretreatment reagent and a measurement FAQD contained in a color-developing reagent. The FAQD used is not particularly limited, and for example, any of a FAQD-a, a FAQD-6, and a FAQD-a may be used. Unless otherwise stead, the measuring kit according to the present embodiment has the earne configuration as that of the measuring kit according to Embodiment B-1, and can be used in the measurement of glycated that in the same manner as in Embodiment B-1.

no in the same manner as in Emporation 6-1.

[0216] Since FAODs have properties that they act on glycated amino acids more easily than on glycated proteins, the glycated amino acid is degraded by the degradation FAOD, However, since the same FAOD is used as the degradation FAOD and the measurement FAOD unlike Embodiment 8-1, there exists a problem as follows. That is, if the Glocated Hp is decladed by adding the budgesse teadeut to the bietiestatest continue onlying in the state where the expense the a continuous of naturing the processor requires to any processor the glycated His degradation degradation FAOD still remains, the remaining FAOD reacts with the glycation site of the glycated His degradation

product so that the ghyseted No cannot be measured accurately. Therefore, when using the same FACO, it is necessary product so that the grycated no cannot de measured accurately, i hardrois, when using the serve FADU. It is necessary that the protesse contained in the protesse contained in the protesse contained in the protesse reagent serves not only to degrade the grycated Hb but also to bractivate the degradation FAOU remaining in the color-developing reaction solution so as to prevent the remaining degradation FAOU from reacting with the glycated Hb degradation product. To this end, the around of the protesse contained in the protesse respont needs to be sufficient to brackhorto the degradation FAOD in the protectment reagent rapidly and

to organize the grycates rate.

[0219] The type and the content of the protesse are not perticularly finited, and preferably are determined as appropriate depending on the type and the amount of the FACO used, the substrate specificity of the protesse used with propriate depending on the type and the like of the glycated Hb, the district ratio of the protesse when the protesse respect to the FACO, the amount and the like of the glycated Hb, the district ratio of the protesse when the protesse respect to the FACO, the amount and the like of the glyceted Hb, the deution ratio of the processes when the processes reagent are edded to the reaction solution, etc. Examples of the combination of a FACO and a product named FOO (Asahi Chemical Industry Co., Lid.) and a product named FOO (Asahi Chemical Industry Co., Lid.) and a product named Proteinase K Co., Lid.) and the combination of a FACO derived from the genus Gibberrote and a product named Proteinase K

(Mefiment-La Roche Inc.).

[0220] Specifically, the protease reagent preferably is edded so that the concentration of the protease in the protease [0220] Specifically, the protease reagent preferably is edded so that the concentration of the degradation FACO is 100 U/A. More and particularly preferably 100 to 100,000 KU/A, when the concentration of the degradation FACO is 100 U/A. More support preferably is edded so that the concentration expedifically, when trype is laused as the protease reagent preferably is added so that the concentration of the type in in the protease reaction actually falls in the range from 1000 to 30,000 KU/A, the concentration of blood of the type in in the protease reaction actually falls in the range from 10 to realist table in the cancel from 0.216 5 to 100. cells (dis in the range from 0.216 5 volts, and the concentration of the degradation FACD fells in the range from 10 to 100 U/I, for example, Furthermore, the reaction is carried out, for example, under the conditions as follows; the reaction too on, an example, number made, was reaction to burney out the standard made from 10 minutes to 20 hours; and the pH temperature in the range from 20°C to 50°C; the reaction period in the range from 10 minutes to 20 hours; and the pH

[0221] In the present embodiment, it is necessary to add a sufficient amount of the measurement FAOD because [0221] In the present embodiment, it is necessary to add a sufficient amount of the measurement FAOD contained in the color-developing reagent may also be inactivated there is a possibility that the measurement FAOD contained in the color-developing reagent may also be inactivated.

was the process.

[2222] Specifically, the color-developing reagent preferably is added so that the concentration of the measurement [2223]. Specifically, the color-developing reagent preferably is added so that the concentration of the measurement FAOD treatment is, for example, in the range from 10 to 1,000,000 FAOD in the reaction solution of the measurement FAOD treatment is, for example, in the range from 10 to 1,000,000 FAOD in the reaction solution of the measurement is a solution of the measurement of the measurement is a solution of the measureme U/I, more preferably from 100 to 200,000 U/I, end particularly preferably 500 to 50,000 U/I, when the concentration of

[U223] As the specific conditions of the measurement FAOD treatment, it is preferable that the color-developing toadeut is ledged so that the concentration of the measurement EVOC in the reaction equation talls in the range from resignit is account or that the concentration of the measurement FACO in the reaction solution falls in the range from 500 to 20,000 U/I, the concentration of the professe in the reaction solution falls in the range from 100 to 30,000 KU I, and the concentration of blood cetts in the reaction solution falls in the range from 0.01 to 1 volts. Furthermore, the reaction is carried out, for example, under the conditions as follows: the reaction temperature in the range from 15°C

to 40°C; the reaction period in the range from 1 mirrute to 1 hour; and the pH in the range from 8 to 9.

(Embodiment B-3)

- [0224] The present embodiment is one example of the third measuring lett, in which the same FACO is used as a degradation FAOD contained in a protrestment reagent and a measurement FAOD contained in a color-developing degradation FAOD contained in a color-developing temperature. However, the present embodment differs from Embodiment 8-2 in that it is not always nocessary to inactivate a degradation FAOD contained in the pretreament reagent with a protecte. Because of the substrate specificity of o veground in the contained at the preference to the seguent must be protected. Secured of the FAOD and protected. enzymes, inactivating a FAOD with a protesso can be diricult depending on the committee of the FAOD and protesso. A measuring kit according to the present embodiment is effective in such a case, if the degradation FAOD contained in the pretreament reagent reacts with a glycated Hb degradation product, the occurrent of the mecturement cannot be improved. Accordingly, it is important to adjust the ratio of the FAOD in the pretreatment reagent to the FAOD in
 - [0228] The FAOD used in not particularly limited, and for example, any of a FAOD-o, a FAOD-S, and a FAOD-nS (1222) Into FACU 1230 in not particularly limits, and in value, and to the present embodiment has the same configurated united as that of the measuring kit according to Embodiment 8-1, and can be used in the measurement of glycuted united as that of the measuring kit according to Embodiment 8-1, and can be used in the measurement of glycuted
 - Hb in the same manner as in Embodiment 6-1.

 [1225] Examples of the combination of a FAOD and a protesse falling within the present embodiment include the [VZZD] EXAMPLES OF THE EXPENDITION OF A PACKET AND A PACKET AND A PACKET AND A STAND AND A PACKET AND A STAND AND A PACKET AND A STAND AND A PACKET AND A STANDARD AND A ST
- the genus Gabernise (AHKRAY, INC., JP 8(1956)-154572 A) and a protesso such as a product named Trypain (sigme Chemical Co.) and a product named Proteinase K (Weke Pure Chemical Industrias, Ltd.).

 [0227] The degradation FAOD needs to be added in an emount such that, even if the activity of the degradation FAOD media to be added in an emount such that, even if the activity of the degradation FAOD media to the special to the degradation product tormad. Further, FAOD media during the proteins the treatment, it does not act on the glycated amino acids easily whereas they do not act more, in order to utilize the properties of FAODs that they act on glycated amino acids easily whereas they do not act.

on glycated proteins easily, the amount of the degradation FACO to be added desirably is set so as to allow the deg-

radation FACD to act only on the glycated amino acid.

Taxiation FACD to act only on the glycated amino acid.

Taxiation FACD to act only on the glycated amino acid.

Taxiation FACD to be concentration of the degradation FACD in the pretreatment reagent is, for example, in the range from 10 to 20,000 U/l, pra/orably from 20 to 10,000 U/l, and more preferably from 100 to 5000 U/l. On the other hand, the concentration of the measurement FAOD in the color-developing reagant is, for example, in the range from 0.5 to 200 KU/I, preferably from 1 to 100 KU/I, and more preferably from 2 to 100 KU/I.

[0229] The pretreatment reagent preferably is added so that, in the pretreatment reaction solution, the concentration of the degradation FAOD fells in the range from 10 to 6000 U/I and the concembration of the blood calls falls in the range

[0230] When using this measuring kit, in order to utilize the properties of FAODs that they ext on glycosted emine scids easily whereas they do not act on allocated proteins easily, the reaction period in the treatment is ment respent containing the degradation FACO preferably is act so as to allow the degradation FACO to ect only on International community on degracement PACO preferrory is an as as to enter the degracement PACO to ext only of the glycated sinthe acid, for example. Specifically, the freetment preferably is carried out under the conditions, for example, as follows: the reaction temperature in the range from 20°C to 37°C and the reaction period in the range from 1 minute to 80 minutes; more preferably the creation temperature in the range from 25°C to 37°C and the reaction temperature in the range from 25°C to 37°C and the condition temperature in the range from 25°C to 37°C and the condition temperature in the range from 25°C and the condition temperature in the range from 25°C and the condition desired in the condition temperature in the range from 25°C and the condition desired in the condition temperature in the range from 25°C and the condition desired in the condition temperature in the range from 25°C and the condition desired in the condition temperature in the range from 25°C and the condition desired in the condition temperature in the range from 25°C and the condition temperature in the range from 25°C and the condition temperature in the range from 25°C and the condition temperature in the range from 25°C and the condition temperature in the range from 25°C and the condition temperature in the range from 25°C and the condition temperature in the range from 25°C and the condition temperature in the range from 25°C and the condition temperature in the range from 25°C and the condition temperature in the range from 25°C and the condition temperature in the range from 25°C and the condition temperature in the range from 25°C and the condition temperature in the condition tempe

person in the realige from a residual to 50 hours, and personality in the condition of the reaction period in the range from 3 minutes to 10 hours.

30°C to 97°C and the reaction period in the range from 3 minutes to 10 hours.

[0231] In the present ombodiment, the prefrontment reagent and colon-developing reagent professibly is added so treat] in the present anabouncers, the pretreament reagant and color-developing reagant pretrably is added so that the ratio (activity ratio G: H) of the degredation FAOD (G) to the measurement FAOD (T) it is 10. So, and particularly reaction solution is, for example, in the range from 1:3 to 1:100, preferably from 1:10 to 1:40, in the present embodiment, the degredation FAOD remains in the protesse reaction. primary from 1: 10 to 1: 40, in the present embouring, the degrated range, the remaining degradation collision unlike Embodiment B-2. However, when the ratio is in the above-described range, the remaining degradation FAOD does not ect on the glycated His degredation product during the protesse treatment to such an extent that it affects the measurement because the reaction rate of the remaining degredation FAOD is very low.

[0232] The present embodiment is an example where DA-64 is used as a color-developing substate contained in a color-developing reagent. Unless attended stated, the measuring kit according to the present embodiment has the same configuration as that of the measuring kit according to Embodiment B-1, and can be used in the measurement

[0233] When the protesse resignat contains a terresolium compound and sodium aside to improve the measurement of glycated Hb in the same manner as to Embodiment B-1. (UZZ-) when the protegne resignit contains a terrecolum compound and social action to improve the measurement sensitivity as described above and the color-developing respect contains DA-64 as a color-developing substrate, the above-described color development error of the DA-64 may occur due to the presence of the tates other compound and the social action action action in the color development the color development action action. Therefore, in order to provent the color development action action.

religiment error, the respective resignitic proferably have the following compositions.

development arror, the respective resigents preferably have the following compositions.

[10234] It is preferable that at least one of the pretreatment reagent, the protesso reagent, and the color developing reagent contains a surfactant. As the surfactant, the above-described surfactants can be used, for exemple.

[10235] When the protessment reagent contains a surfactant, the concentration of the surfactant is in the range from 0.01 to 50 mmol/l, when the protesse reagent contains a surfactant, the concentration of the surfactant is in the range from 0.01 to 50 mmol/l, preferably 0.05 to 20 mmol/l, when the color-developing reagent on the surfactant, the concentration of the surfactant is in the range from 0.01 to 50 mmol/l, preferably 0.05 to 20 mmol/l, when the color-developing reagent contains a surfactant, the concentration of the surfactant is in the range from 0.06 to 30 mol/l, preferably 0.1 to 20 mol/l.

[10236] The respective respents of the measuring kit may be added so that the concentration of reoperties components in the color-developing reaction solution fall within the following ranges. Preferably, the concentration of: the DA-64 is 0.001 to 100 mmol/l, the surfactant is 0.002 to 50 mmol/l, the strizeofium compound is 0.1 to 10 mmol/l, the surfactant is 0.002 to 50 mmol/l, the strizeofium compound is 0.8 to 8 mmol/l; the surfactant to 0.02 to 10 mmol/l; the strizeofium compound is 0.8 to 8 mmol/l; the surfactant to 0.02 to come to continuous and sequencement compound as one to a minious, and the surfactant to 0.02 to 10 minous, the totraxolum transports the concentration of: the DA-64 to 0.01 to 1 minous, the surfactant to 0.02 to 10 minous, the totraxolum compound is 0.7 to 2.7 mmoV; and the sodium saide is 0.2 to 1.5 mmoV.

[0237] The pH of the color-developing reaction solution preferably to in the range from 6.0 to 9.0, more preferably [0258] The DA-64 develope color by the reaction. Thus, by measuring the absorbance (i.e., the degree of the [0258]. The DA-64 develope color by the reaction that reaction color developed) of the reaction colorion with a spectrophotometer, for example, at a wavelength in the range from 850 to 750 nm, the amount of the hydrogen peroxide can be determined. from 6.5 to 8.5, and particularly preferably from 7.0 to 8.0.

FXAMPLES

(Example 1)

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[0239] Whole blood samples were collected from patients (the number of patients: 163), and the erythrocytes in the emples were atomed to prodpliste naturally and collected. Thereafter, 10 µl of the arythrocyte fractions were mixed with 600 µl of the following protreatment reagent to prepare hemolyzed samples (n = 163). Since the collected crythroid to produce the following protreatment reagent to prepare hemolyzed samples (n = 163). rocyto tractions were obtained by causing anythrocytes to precipitate naturally, the homolyzed samples also contained components in plasma.

(Pretrantment Reegant: pH 9.4)		
CHAPS Surfectant (product name NIKKOL-SLIPEX, Nikko Chemicale Co. Ltd.)	Nemm 08 Ng 9	
FACO (product name FACX, Mikkoman Corporation)	1 KU/	

[0240] Next, 20 μ of the respective homolyzed samples were mixed with 78 μ of the following protesse reagent, rest, ω μ or the respective nemoryton samples were mixed with to the or the following processe reagain, and the resultant mixtures were incubated at 37°C for 5 minutes. Subsequently, 19 μ of the following color-developing and the resultant mixtures were incubated at 37°C for 3 minutes. Then, the absorbance reagant further was added, and the resultant mixtures were incubated at 37°C for 3 minutes. Then, the absorbance was measured at the wavelengths of 761 nm and 671 nm. For the measurement of the absorbance, an automatic samples appearable (product name JCA-8M 8, manufactured by Japan Electron Optics Laboratory Co. Ltd.) was used.

(Protesse Reagent: pM 5.5) Tetrezofium compound (product name WST-9, Dojindo Laboratorica) NBN ₃ NACI CACL ₂ NBURTH Protesse (ARKRAY, INC.) MES	2 mmot/l 0.8 mmot/l 100 mmot/l 2 mmot/l 4 MU/l 1 mmot/l
(Color-Developing Reagent: pH 7.0)	
Product nemo DA-84 (Wako Pure Chemical Industries, Ltd.) FAOD (ARKRAY, INC.) Tria-HCI NaNg	36 mms/ 380 mms/ 0.5 mms/

[0241] Thereafter, the thus-measured absorbances were substituted into previously prepared calibration curves showing the relationships between a Hb concentration (y/I) and absorbance and between a HbA1c concentration (y/I) and absorbance and between a HbA1c concentration (y/I) and absorbance and between a HbA1c concentration of I) and absorbance, respectively, to determine the Hb concentration and the HbA1c concentration. Then, HbA1c's was calculated using the following equation. The Hb concentration can be determined based on the obserbance measured at the wavelength of 751 nm, and the HBA1c concentration can be determined based on the absoluence measured at the wavelength of 571 nm.

HbA1c (%) = (HbA1c concentration / Hb concentration) \times 100

[0242] The calibration curves were prepared in the following manner. First, standard solutions with various known concentrations of kib were provided. Then, the HbA1c concentration and the Hb concentration of these standard seconcentrations of No were provided. Then, the HbA1c concentration and the Hb concentration of thiss standard so-lutions were megalized by HPLC using a HbA1c measuring appearable (product name HA-916c, manufactured by ARKRAY, INC.). On the other hand, with respect to these standard solutions, the obsorbance corresponding to the HbA1c concentration were measured in the same manner as concentration and the secondarics corresponding to the HDA1c concentration were measured in the same mainter as described above. Based on the measured values given by the automatic analysis apparatus and the absorbances thus measured, primary regression equations were prepared, which were used as the calibration curves.

[0283] Furthermore, the value obtained by performing the measurement by HPLC method with respect to the hemo-

lyzed samples (n = 163) prepared in Example 1 was used at a control.

[0244] The results of the above-described measurements are shown in FIG. 1. FIG. 1 is a graph showing the rela-

Bonship between the HBA1c (%) in Example 1 measured by the enzymatic method and the HBA1c (%) as the control obtained by HPLC, in Fig. 1, Example 1 has a correlation equation of "y = 1.031x - 0.03" and a correlation coefficient

[0245] Thus, the value obtained in Example 1 was very close to the control value. Furthermore, Example 1 exhibited 6 the axtended high correlation coefficient (0.981) with the control. Therefore, it is understood that glycated Hb was measured with high occursor.

Industrial Applicability

10 [0248] As specifically described above, the method of measuring a shocked protein and the measuring kit according to the present invention can achieve excellent measurement accuracy because the influence of a glycated amino acid present in a sample with the glycated protein as an analyte on the measurement can be eliminated. Accordingly, by applying the present invention to, for example, the measurement or glycated Hb and HbA1c in erythrocytes, more reliable measured value can be obtained than by conventional methods, which further increases the importance of the glycated Hb and HbA1c as indicators in the diagnosis and the like of diabetes.

Claims

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A method of measuring an amount of a glycated protein in a sample, comprising:

causing a fructosyl amino acid oxideae to act on a glycated emino acid present in the sample other than the glycated protein so as to remove the glycated amino acid by degrading it; then causing a fructosyl amino acid oxideae to act on the glycated protein to cause a rodox reaction in the presence of a tetracellum compound and social marklo; and managing the redox reaction to determine the amount of the glycated protein.

- 2. The mothod according to claim 1, wherein the glyceted protein is glyceted hemoglobin.
- The method according to claim 1, further comprising:

degrading the glyceted protein with a protesse to give a degradation product of the glyceted protein elther before or after causing the fructosyl arrino acid oxidesia to act on the glyceted smilno acid,

- 35 wherein the fructoryl amine acid exidase caused to act on the glycated protein is caused to act on the degradation product to cause the reaction.
 - The method according to claim 1, wherein the measurement of the redox reaction is measurement of an amount
 of hydrogen peroxide formed by causing the fructoryl amino acid oxidese to act on the glycated profein, the measurement of the amount of the hydrogen peroxide comprising:

adding N-(carboxymethylaminocarbonyi)-4,4-bib(draethylamino) diphonylamine socium sait as a color-developing substrate to a reaction solution of the redox reaction in the presence of a surfactant, thereby causing a redox reaction between the color-developing substrate and the hydrogen peroxide; and

measuring the amount of color developed by the color-developing substrate to determine the amount of the hydrogen peroxide.

wherein, a concentration of the tetrazelium compound in the reaction solution is in a range from 0.5 to 8 mmoV, a concentration of the sodium azide in the reaction solution is in a range from 0.08 to 0.8 mmoV, a concentration of the surfactant in the reaction solution is in a range from 0.3 to 10 mmoV, and a pH of the reaction solution is in a range from 7.0 to 8.5.

- 5. The mathod according to claim 1, wherein the fructoryl amino acid exidese caused to act on the glyceted amino acid is specific for a glyceted α-amino group, and the fructoryl amino acid exidese caused to act on the glyceted protein is specific for a glyceted α-amino group and a glyceted side chain of an amino acid residue.
- The method according to claim 1, wherein a solution confining the tetrazollum compound and the sodium exide is aged and is then added to the cample.

- The method according to claim 1, wherein the tetrazolium compound & 2-(4-loduphanyt)-3-(2 /-dinitrophanyt)-6-(2,4-disultophanyt)-2H-tetrazolium sait.
- 8. A method of determining a ratio of glycenod hemoglobin to hemoglobin, comprising:

measuring an amount of glycated homoglobin in a sample by the method according to claim 1; measuring an amount of homoglobin in the sample; and calculating the ratio of the glycated homoglobin to the homoglobin using the amount of the glycated homoglobin and the amount of the homoglobin thus measured.

A measuring kit used for measuring a glycetod protein using a redox reaction, comprising:

a pretreatment reagent for protreating a sample, contribing a fructoryl amino acid oxidese; and a color-developing reagent containing a fructoryl amino acid oxidese, an exidereductase, and a color-developing substrate.

- 10. The measuring kill according to claim 9, wherein the glycated protein is glycated hemoglobin.
- 11. The measuring kit according to claim 9, wherein the hydroxyl amino acid oxidase contained in the pretreatment reagent is specific for a giyested a-emino group, and the fructosyl amino acid oxidase contained in the color-developing reagent is specific for a giyested a-emino group and a giyested side chain of an amino acid residue.
 - 12. The measuring kit according to claim 9, further comprising a protesse reagent containing a protesse.
- 23 13. The measuring lot according to deim 12, wherein the protease is at least one protesse enlected from the group consisting of metalloproteinsess, prometals, papels, trypels, proteinses K, subtiliels, and aminopeptidess.
- 14. The measuring lot according to claim 12, wherein the proteins is at least one proteins that degrades glyceled hemoglobin selectively and is eslected from the group constraing of metalloproteinnaces, bromekin, papain, trypain derived from poreine pancress, and proteins derived from Beating aubities.
 - The measuring kit seconding to claim 12, wherein the proteins reagent further contains a tetrazellum compound and sedium azide.
- 18. The measuring lift according to claim 16, wherein, in the protease reagant, the totrazollum compound (A) and the sodium exide (B) are present at a ratio (moter ratio A : B) in a range from 20 : 3 to 20 : 12.
 - 17. The measuring kit according to claim 12, wherein the problems regent contains a metalloproteinness as the protessas and further contains Cq and Na, and a concentration of the metalloproteinness to in a range from 100 to 40,000 U/I, a concentration of Ca is in a range from 0.1 to 50 mmoVI, and a concentration of Na is in a range from 5 to 1000 mmoVI.
 - 18. The measuring kit according to claim 9, wherein the coten-developing substrate is N-(carboxymethylaminocurbo-nyl)-4,4-big(dmothylamino) diphonytemine sodium 998.
 - The measuring kit eccording to claim 9, wherein at least one of the protreatment reagain and the color-developing reagant further contains a surfactant;
 - 20. The measuring kit according to claim 12, wherein the protesse reagent further contains a surfactant.
 - 21. The measuring kit according to claim 19 or 20, wherein the surfactant is at least one surfactant adjected from the group consisting of polyoxyethylene others, polyoxyethylene phenol others, polyoxyethylene acrottan alkyl exters, and polyoxyethylene alkyl others.
- 22. The measuring kit eccording to claim 9, wherein the pretreatment reagent further contains at least one buffer selected from the group constating of CHES, MOPS, TAPS, EPPS, phosphate, HEPPSO, POPSO, and borate, and a pit of the protreatment congent is in a range from 8.0 to 10.0.

- 23. The measuring kit according to claim 9, wherein the color-developing reagent further contains at least one buffer selected from the group constating of MES, Tris, phosphate, MOPS, TES, HEPES, HEPPSO, and EPPS, and a pH of the color-developing reagent in a range from 6.0 to 9.0.
- 24. The measuring kit according to claim 12, wherein the protesse reagont further contains at least one buffer colocard from the group consisting of Trie, MES, DIPSO, TES, POPSO, HEPES, MOPSO, Bla-Tria, MOPS, ADA, PIPES. ACES, and phosphate, and a pH of the protesse reagent is in a range from 5.0 to 7.0.
- 25. The measuring kit according to claim 15, wherein the trinizolium compound is 2-(4-todephenyl)-3-(2,4-dinitrophenyl)-6-(2,4-dinitrophenyl)-2H-terrezolium salt.
 - 28. The measuring kit according to claim 9, wherein the pretreatment reagont further contrins at least one of urbases and billrubin oxidesa.
- 27. The measuring kit according to claim 9, wherein the color-developing reagent further contains sodium saids.
 - 28. The measuring kit according to cinim 22, wherein

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the fructoryl amino acid coddase in the protrestment reagent is specific for a glycated a-amino group; in the pretractment reasent, a concentration of the fructional surface acid exists a line in a range from 10 to 5000 and a concentration of the buffer is in a range from 5 to 200 mol/l; and a pH of the pretrantment reagent is in a range from 8.0 to 10.0.

- 29. The measuring kit according to claim 15, wherein
 - the protesso regent further contains Co, Na, and a buller;
 - the protects in the protecte regard is a metalloproteinase;

In the proteose regent, a concentration of the metalloprotein/aso is in a range from 100 to 10,000 KU/L a concentration of the tetrazellum compound is in a range from 0.1 to 10 mmot/l, a concentration of the sedium azide is in a range from 0.08 to 4 mmol/l, a concentration of Ca is in a range from 0.1 to 50 mmol/l, a concentration of Na is in a range from 5 to 1000 mmoV, and a concentration of the buffer is in a range from 0.1 to 500 moV; and a pH of the protosso reagent to in a range from 5.0 to 7.0.

- 30. The measuring lot eccording to daim 23, wherein,
 - In the color-developing respect, the fructional amine sold exidence is appealed for a glycated common group and a glycated side chain of an amine sold residue, the exidence developing and a glycated side chain of an amine sold residue, the exidence developing
 - substrate is N-(carboxymethylaminocarboryl)-4,4-bis(dimethylamino) diphenylamino sodium selit, in the color-developing reagent, a concentration of the fructoryl emino acid oxidase is in a range from 100 to 50,000 U/I, a concentration of the perceideso is in a range from 0.1 to 400 KU/I, a concentration of the N-(car-bosymethylaminocarbonyl-4,4-bis[directhylaminosid]bhernylamino sodium salt is in a range from 0.02 to 2 mmoV It and a concentration of the buffer is in a range from 10 to 500 mol/l; and
- B pH of the color-developing reagent is in a range from 6 to 9.

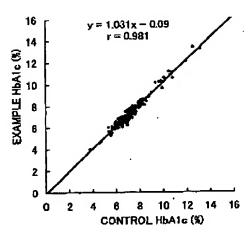


FIG. 1

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